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The production, characterization and application of monoclonal antibodies specific for *Pseudomonas syzygii*, the causative agent of Sumatra disease of cloves

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THE PRODUCTION, CHARACTERIZATION AND APPLICATION OF
MONOCLONAL ANTIBODIES SPECIFIC FOR PSEUDOMONAS SYZYGII,
THE CAUSATIVE AGENT OF SUMATRA DISEASE OF CLOVES

Submitted by David John Ambler
for the degree of PhD
of the University of Bath
1990

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SUMMARY

Polyclonal antisera raised against Pseudomonas syzygii, the causative agent of Sumatra disease of cloves in Indonesia, had been found to cross-react extensively with Pseudomonas solanacearum. As well as revealing a previously unexpected relationship, this finding was also important as it prevented reliable serological diagnosis due to the occasional presence of P. solanacearum in clove trees.

This thesis describes the production of a specific serological reagent consisting of a mixture of seven P. syzygii specific monoclonal antibodies (mAbs).

Detection of P. syzygii in clove wood was demonstrated using the mAbs in both dot-immunobinding and immunofluorescence assays (IFA).

The potential of both DAS-ELISA and IFA for the detection of P. syzygii in its insect vectors was also demonstrated.

The basis for the serological cross-reactivity between P. syzygii and P. solanacearum was shown to be mainly due to the presence of antigenically related lipopolysaccharide on the surface of both species.

A panel of five mAbs with variable reactivity both within and between the two species was used to identify serogroups of P. syzygii. The serogroups correlate well with the geographical origin of the isolates within them.

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APPENDIX I - REAGENTS USED AND SUPPLIERS

APPENDIX II - BUFFERS, MEDIA, ETC

ABBREVIATIONS USED IN THE TEXT

>	greater than
<	less than
A ₄₅₀	absorbance at 450 nm
Ab	antibody
AFRC-IACR	Agriculture and Food Research Council Institute of Arable Crops Research
AFRC-IAPGR	Agriculture and Food Research Council Institute of Animal Physiology and Genetics Research
Ag	antigen
ALP	alkaline phosphatase
AY	aster yellows
BCYE	buffered charcoal yeast extract medium
BDB	banana blood disease bacterium
BLO	bacteria-like organism
CA+	casamino acids medium (iron supplemented)
CM	cytoplasmic membrane
4CN	4-chloro-1-naphthol
DAB-ELISA	direct antigen binding enzyme-linked immunosorbent assay
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DIA	dot immunobinding assay
DMEM	Dulbecco's modification of Eagle's medium
DMP	dried milk powder
EM	electron microscope
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	fluorescein isothiocyanate
g	gram
x g	x gravity
GA	glutaraldehyde
H	hypoxanthine
ha	hectare
HAT	hypoxanthine/aminopterin/thymidine
HAza	hypoxanthine/azaserine
HRP	horseradish peroxidase

HT	hypoxanthine/thymidine
IFA	immunofluorescence assay
Ig	immunoglobulin
i.p.	intraperitoneal
ISEM	immunosorbent electron microscopy
i.v.	intravenous
kDa	kilodalton
km	kilometre
KOH	potassium hydroxide
LPS	lipopolysaccharide
mAb	monoclonal antibody
MLO	mycoplasma-like organism
M ϕ	macrophage
MW	molecular weight
ND	not determined
OD ₆₅₀	optical density at 650 nm
ODA	Overseas Development Administration
ODD	Ouchterlony double diffusion
OM	outer membrane
pAb	polyclonal antibody
PBS	phosphate buffered saline
PDB	Pierce's Disease bacterium
PEG	polyethylene glycol
pv.	pathovar
PW	periwinkle wilt medium
RIA	radioimmunoassay
R-Pe	R-pycoerythrin
RSD	Ratoon stunting disease
SD	Sumatra disease (of cloves)
SDB	Sumatra disease bacterium
SD.SM	Sumatra disease (bacterium) specific mixture (of mAbs)
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
subsp.	subspecies
TRITC	tetramethylrhodamine isothiocyanate
XLB	xylem-limited bacterium

CHAPTER 1

GENERAL INTRODUCTION

1.1. SUMATRA DISEASE OF CLOVES AND THE SUMATRA DISEASE BACTERIUM

1.1.1. History

The clove tree (*Syzygium aromaticum*) is an important smallholder crop in Indonesia. A major use of cloves is in the manufacture of Kretek cigarettes, which consist of a mixture of shredded cloves and tobacco. During recent years, a mass decline of cloves in Sumatra and Western Java has caused widespread devastation (Bennett et al; 1987).

The decline is mainly the result of Sumatra Disease (SD), a term first used by Waller and Sitepu (1971) who recognised that the mass decline then active in Sumatra was a pathogenic state.

SD affects the vascular system with symptoms of progressive leaf fall starting in the upper crown, leading to tree death in 1-4 years. Annual crop losses due to SD have been estimated to be in the region of £10 million (Eden-Green, 1988).

In 1975 a co-operative clove disease research project (Project ATA-71) between Indonesia and the United Kingdom was initiated under the Colombo plan. Early work under this project confirmed that SD was the result of an infectious agent.

The consistent association of a bacterium with SD was first reported by Bennett et al. (1985). Light microscopy of sections cut from the greyish-brown discoloured wood of diseased trees revealed the consistent presence of small rod-shaped bacteria within the xylem vessels, such invaded vessels being easily detected following trypan blue

staining.

Electron microscopy of invaded xylem vessels showed the bacterium to be rod-shaped with rounded ends, 0.5-0.6 μm x 1.0-1.5 μm (occasionally up to 2.5 μm long). The cell wall, which is 25-50 nm thick, has a typical Gram-negative structure, comprising an inner and an outer membrane between which is a layer of varying density and thickness. Neither fimbriae, flagella nor capsules were observed (Bennett et al., 1987).

Axenic culture of the bacterium was eventually achieved in 1979 (Bennett et al., 1987), who found that three conditions had to be met before isolation from infected clove wood was possible; namely, the use of freshly gathered infected wood, the squeezing of the bacteria to the cut surface by mechanical pressure (i.e. pliers), and the immediate removal of inoculum to a culture medium of low ionic strength.

The isolated, nutritionally fastidious bacterium could not be classified into any known genus and was given the colloquial name "Sumatra Disease Bacterium" (SDB).

The pathogenicity of the bacterium was demonstrated by inoculation to the xylem of clove seedlings and young trees with a suspension of cultured bacteria. Over 60% of the plants died within two years, Koch's postulates being fulfilled by the re-isolation of the same bacterium from them (Hunt et al. 1987).

Primary spread of SD is characterized by jumps of several kilometres followed by secondary spread within affected clove gardens, a pattern which suggested an airborne vector (Eden-Green et al; 1987a). In addition, ultrastructural similarities and the fastidious nutritional requirement of SDB suggested a possible relationship with other nutritionally fastidious, vector-borne, xylem-limited bacteria causing serious plant diseases (e.g. Pierce's disease of grapevine), which are now grouped in the genus *Xylella* (Wells et al., 1987).

This hypothesis, although now known to be incorrect, led, by analogy, to the identification of the tube-building cercopoids (xylem-feeding Homoptera) Hindola striata in Java and Hindola fulva in Sumatra as vectors of

SD (Eden-Green et al., 1987a & 1987b).

An unexpectedly close serological relationship between SDB and Pseudomonas solanacearum, in contrast to major morphological and cultural differences, was observed by Eden-Green et al. (1987c), casting doubts upon the reliability of serological diagnosis since P. solanacearum is occasionally isolated from clove trees. This finding caused additional complications in that some workers in Indonesia considered P. solanacearum to be the causal agent of SD (Hidir, 1973; Machmud, 1987), based on its isolation from the roots of clove trees with SD symptoms and the wilting of clove seedlings upon re-inoculation.

Isolation of P. solanacearum (probably present either as a limited invasion resulting from root damage or as a secondary infection) in such cases is probably due to the use of isolation media which favour the identification of the faster growing P. solanacearum over that of the slow growing SDB. While some P. solanacearum isolates are pathogenic to clove seedlings, and P. solanacearum can occasionally be recovered from the roots of mature trees, only SDB has been observed to cause the systemic invasions of xylem vessels found in diseased trees (Eden-Green & Machmud, 1988).

The requirement for the clarification of the taxonomy of SDB and its relationship to P. solanacearum led to the inception of ODA research project R4130 at Rothamsted Experimental Station, with the additional aim of producing a specific diagnostic test for SDB.

Two workers were involved in the project, Dr S.J. Roberts and myself (D.J. Ambler).

As a result of this work, SDB was determined as being a new species of the genus Pseudomonas, namely Pseudomonas syzygii (Roberts et al; 1990). This name will be used from now on within this text.

1.1.2. Symptoms

Two syndromes of SD have been observed, a rapid decline and a slow decline, the former being the most widespread (Bennett et al; 1985). The observation of intermediate symptoms suggests that they are manifestations of the same disease, however, the factors determining the symptom pattern have not been identified.

Death of all trees exhibiting rapid decline symptoms occurs three months to two years after their appearance. The first symptom is usually a sudden leaf fall and twig dieback at branch tips near the top of the tree, leaf loss mainly progressing downwards for several weeks or months.

Leaves generally pass through a transitory chlorotic and yellow phase before falling but occasionally on some branches (or the whole tree) the leaves may be affected so rapidly that they wilt, turn brown and remain attached to the tree. As the progressive and total leaf fall is occurring in the crown, leaves lower in the canopy become pale green and lack-lustre. Progressively more and more leaves are lost, more branches die back and the whole tree dies.

The root system also deteriorates at the same time, loss of some fine feeder roots occurring before aerial symptoms. Progressive degeneration of more and larger roots occurs, often accompanied by secondary brown or greyish-black internal fungal rots.

Cutting the wood obliquely reveals greyish-brown longitudinal streaks mostly in the youngest wood near to the cambium. The streaking is most abundant in the roots and trunk base and can usually be traced right up into the affected branches at the tree top, even at the onset of symptoms. The discoloured streaking becomes more extensive as symptoms develop, and liberates a milky bacterial ooze.

Typical symptoms of the slow decline syndrome are a general chlorosis and leaf loss, affecting all parts of the tree more or less simultaneously. Sudden total leaf loss from the upper branches and leaf wilting have not been observed. Periods of leaf fall alternate with periods of partial recovery. Trees are eventually weakened and die, up to five years after first symptoms.

Plate 1.1.1 compares a healthy clove tree and a clove tree exhibiting SD symptoms (i.e. leaf fall and progressive die back from the top). Plate 1.1.2 shows the devastating effect of SD on clove plantations.



Plate 1.1.1

The tree on the left is exhibiting symptoms of SD, whereas that on the right is apparently healthy.



Plate 1.1.2

The effect of Sumatra disease on a clove plantation. All of the trees are either dead or dying.

1.1.3. Epidemiology

In the case of rapid decline, all trees older than about three years in a clove garden are attacked and die over a period of four to five years. In gardens of mixed age trees, the oldest are usually the first to become affected followed by progressively younger trees, with only seedlings and young trees under three years old rarely developing symptoms (Bennett et al; 1985).

The fact that much of the pathogenicity testing of P. syzygii on cloves was done using seedlings under two years old (Hunt et al; 1987) at first appears to contradict the previous statement. The optimum conditions for the successful establishment of bacterial infection in seedlings were found to be: the requirement for vigorously growing seedlings, inoculation of the stem base by needle puncture, and incubation under 50% shade in a cool (<30°C) environment (Hunt et al, 1987). The authors suggested that the lack of shade of underlying branches of immature trees in the field would regularly allow heating of exposed branches to above 32°C (the maximum temperature supporting in vitro growth of P. syzygii (Bennett et al, 1987)), thus resulting in the failure of P. syzygii to establish infection. The fuller canopy of older trees may be expected to provide more favourable conditions for bacterial growth.

The disease advances at 1 km or more a year, with local foci of infection occurring beyond the general front and pockets of unaffected trees remaining, for a short time, behind it, survival of mature trees only rarely occurring. Soil type and cultural practices, good or bad, have no significant impact on disease spread. Such observations are indicative of the infectious nature of SD.

The spread of disease within individual gardens is consistent with an air(vector)-borne pathogen (rather than soil-borne spread) in that it spreads equally rapidly uphill or downhill and across streams, with little evidence of centrifugal spread from infected trees (Eden-Green,

1988).

Spread is less rapid and aggressive in the slow decline syndrome, but the end result is the same (i.e. all trees become affected and die).

1.1.4. Distribution of Bacteria in Infected Material

Examination by light microscopy of sections cut from greyish-brown streaked portions of wood from SD affected trees reveals the presence of bacteria within the xylem vessels. The bacteria are confined to the xylem vessels, with affected vessels normally being blocked throughout their length (Hunt et al, 1987).

In heavily affected wood, almost all vessels are blocked by gelatinous bacterial plugs; in less affected wood, blocked vessels are fewer, being scattered or grouped into rings or arcs in the wood, usually at or near the cambium.

The percentage of vessels occluded by bacteria at the time of death of experimentally inoculated seedlings was found to be closely related to the time of incubation (Hunt et al, 1987). 5-10% vessel occlusion was sufficient to kill some plants quickly while others, with slowly developing symptoms, survived with 40% or more of their vessels occluded.

The death of seedlings with less than 10% of vessels occluded may suggest that SD symptoms are not entirely due to mechanical blockage (as was suggested by Bennett et al, 1985). The low percentage observed is not due to discontinuous plugging of vessels as was the case with Pierce's disease (Hopkins, 1981).

Bacteria can usually be seen even in samples taken from trees exhibiting early symptoms, their detection becoming more certain as aerial symptoms progress.

Symptomless trees in affected gardens are usually free from bacteria but bacteria can occasionally be seen in root

and trunk tissue, such trees being presumed to be incubating the disease.

Trees exhibiting slow decline symptoms contain similar occluded vessels.

Whereas P. syzygii is easily isolated from trees at the early stages of the disease, isolation is rarely successful from trees at a late stage of disease or from those with slow-decline, despite the presence of large numbers of bacteria. This suggests that the proportion of viable bacteria in long-infected tissues and in slow-decline trees is low (Bennett et al, 1987).

1.1.5. Distribution of Sumatra Disease in Indonesia

Extensive surveys of Sumatra have shown that SD is widely distributed in all provinces (see Figure 3.1 for a map of Sumatra and Java) with the possible exception of Aceh, Riau and South Sumatra, and is actively spreading in Lampung and North Sumatra. SD is also present in West and Central Java, but is less of a threat in the latter region due to low populations of the vector. Surveys of the other clove growing areas, namely North and South Sulawesi and East Java did not identify any outbreaks of SD (Eden-Green, 1988).

Sumatra Disease has not been reported from any other clove growing countries. Sudden Death disease of cloves (also abbreviated to SD!) which occurs in Zanzibar and Pemba is unrelated to Sumatra Disease and is thought to be caused by a phloem-limited mycoplasma-like organism (Dabek et al. 1985).

1.1.6. The Vector

Since initial work on SD had suggested similarities with diseases caused by XLB of the Pierce's disease group, a search was made, by analogy, amongst xylem-feeding Homoptera associated with clove trees for a possible vector.

Sampling for clove associated insects in W.Java, Lampung and W.Sumatra using direct observations, sticky board traps hung in the clove tree canopy, and sweeping from undergrowth, revealed low numbers of tube-building cercopoids (Machaerotidae) (Hindola striata in W.Java and H.fulva in Sumatra) breeding on clove trees. Nymphs of both species develop in calcareous tubes formed on the surface of young leaf midribs, petioles, and shoot tips. These species were not found on other plants or undergrowth (Eden-Green et al, 1987a).

Little is known about the life cycle of Hindola or other Machaerotidae, and this appears to be the only reported instance of the group having any economic significance (Eden-Green et al, 1987a).

In transmission tests, most plants exposed to Hindola spp. developed typical SD symptoms after maximum incubation periods (post exposure) of 4-12 months. The plants were systemically infected with bacteria which were identified as P. syzygii upon culturing. No control plants, either exposed to other insect species, or kept insect free developed SD (Eden-Green et al., 1987a & 1987b).

Approximately 4% of the natural population of Hindola spp. was estimated to be infective. Isolations of P. syzygii from insects suggested counts of 10^3 - 10^5 bacteria per insect (Eden-Green et al, 1987a).

The results obtained, coupled with the close association of the two species with clove trees confirmed the role of H.striata (in Java) and H.fulva (in Sumatra) in the transmission of SD, and strongly implicated them as the most important natural vectors of P. syzygii.

1.1.7. Diagnostic Methods

Preliminary diagnosis of SD is generally based on symptom expression coupled with macroscopic examination of wood (both from twigs and roots) for streaking and bacterial ooze in order to confirm the presence of a bacterial pathogen (Dr S.J. Eden-Green, personal communication).

Further testing may include microscopical examination of trypan blue stained sections to observe bacterially occluded xylem vessels, and isolation of bacteria onto culture medium.

P. syzygii colonies on isolation plates are generally distinguishable from other bacteria due to their slow growth.

Immunofluorescence and slide agglutination, using rabbit antisera to P. syzygii were successfully used to detect the presence of bacteria in infected wood and to confirm the identity of bacteria from isolation plates until the specificity of the antisera was cast into doubt after the discovery of the serological relationship between P. syzygii and P. solanacearum (Eden-Green et al., 1987c).

Rabbit antisera to both heat fixed (100°C for 1 hour) and glutaraldehyde fixed whole cells of P. syzygii and P. solanacearum cross-reacted between both bacteria in immunofluorescence (IFA) and microagglutination tests.

Cross absorption of anti-P. syzygii antisera with whole P. solanacearum cells removed the cross-reacting fraction of the antiserum but also reduced homologous titres in IFA test on bacterial cultures. The cross-absorbed antisera reacted weakly against P. syzygii in plant sections and were not suitable for diagnostic use.

Apart from direct isolation of bacteria, no reliable method of routinely detecting P. syzygii in the insect vector is available.

1.1.8. Control Measures

Amongst possible control measures for SD three strategies have been investigated by the ODA research team in Indonesia (Eden-Green, 1988):

i). Chemical control: Early investigations into the etiology of SD showed that repeated infusions of oxytetracycline into the wood of initially symptomless clove trees exposed to natural infection by SD resulted in a delay of symptom onset and slower rates of symptom development when compared with untreated trees (Hunt et al. 1985). However, the practical problems involved in maintaining the frequent (4-6 monthly) infusions required to maintain symptom remission or disease protection precluded the use of antibiotics as a short term control measure except for possible small-scale use on particularly valuable trees;

ii). Disease resistance: All commercial clove types are thought to derive, ultimately, from the north and central Moluccan islands. Since none has shown SD resistance during 50 years of exposure to the disease in Sumatra, and also due to the lack of selection pressure in the Moluccas (where SD is thought to be absent), it is thought that there is little chance of finding resistance there.

To date, 34 Myrtaceous species have been tested for SD resistance, of which approximately half showed high levels of resistance or apparent immunity to infection (Pool et al, 1986). Some of these were successfully approach grafted with cloves, P. syzygii does not colonise the resistant rootstock, but is still able to invade and kill the clove scion;

iii). Vector control: The identification of the insect vector of SD, coupled with the problems encountered with other potential control methods, suggests vector control to be the best immediate candidate for control of SD.

Effective control of SD would probably require a large reduction in Hindola populations over a wide area. Control measures would probably only need to be targeted at clove trees since they are probably the only host of any

significance in most areas, and also the immobile xylem-feeding nymph stages should be susceptible to xylem-mobile systemic insecticides.

Studies are presently in progress in Indonesia aimed at further understanding the biology, population dynamics and vector properties of Hindola, which coupled with insecticide screening trials should produce an effective control strategy (Eden-Green, 1988).

The availability of a rapid and reliable diagnostic test would both assist the investigation of the distribution of P. syzygii amongst the vector population as well as allowing the identification of foci of infection which would allow the targeting, rather than the indiscriminate use of, insecticides.

1.1.9. Description of P. syzygii

The results of a detailed characterization of SDB, leading to the proposal of the name Pseudomonas syzygii (sy.zigi.i. L. n. syzygium, generic name of the clove tree; L. gen. n. syzygii, of the clove tree) have recently been published (Roberts et al. 1989).

A summary of the findings follows: P. syzygii is a Gram negative, aerobic, non-sporeforming, non-capsulate, non-motile aflagellate straight rod with rounded ends (0.5-0.6 x 1.0-2.5 μ m), occurring singly, in pairs or occasionally short chains. P. syzygii grows poorly (colonies <1 mm in 7 days) or not at all on many common bacteriological media, but gives good growth (colonies up to 5 mm in 7-12 days) on buffered charcoal yeast extract (BCYE) , periwinkle wilt (PW) and casamino acids (CA+) media, containing iron salts. Growth on complex media is accompanied by rise in pH. On CA+ colonies are initially translucent, later becoming more opaque, cream, circular, smooth, convex with entire margins and a butyrous to viscid consistency, a finely granular internal structure is sometimes apparent. Recovery from agar cultures more than 14 days old is poor. Optimum temperature for growth is around 28°C. No growth occurs at 37°C or 4°C.

Strains vary in their ability to incite a hypersensitive reaction in tobacco.

Isolated as a phytopathogen from xylem tissues of the clove tree (Syzygium aromaticum) and other Syzygium spp., and from insect vectors (Hindola spp.) in Indonesia. The G:C content of the DNA is 66-67 moles% (buoyant density). DNA-DNA hybridisation experiments and fatty acid profiles indicate a close relationship with P. solanacearum and a more distant relationship with P. cepacia. The type strain is R1 (=NCPPB 3446). Can be distinguished from P. solanacearum on the basis of NaCl tolerance, growth at 37°C, growth on common media, carbon source utilisation, fatty acid profiles and pathogenicity on clove and Solanum spp.

The results of the cultural and biochemical tests on 46 isolates of P. syzygii were subjected to numerical cluster analysis. The P. syzygii isolates formed a distinct cluster from P. solanacearum and the other plant pathogenic bacteria included in the tests. The characters differentiating P. syzygii from P. solanacearum are shown in Table 1.1.1. The dendrogram constructed from the results revealed two major groups within P. syzygii, differentiated mainly on D-fructose utilisation. The two major groups were further subdivided into two sub-groups by utilisation of L-asparagine, L-histidine, citrate and α -ketoglutarate and growth on 0.5% NaCl containing medium. Some correlation was noted between the groups and the geographical origin of the isolates within them, i.e. all Javan isolates were contained within group 2, and group 1 only contained Sumatran isolates. The majority of Sumatran isolates in group 2 came from southern Sumatra.

The total cellular fatty-acid composition of selected P. syzygii isolates was determined. Two distinct profiles within P. syzygii were revealed which correspond to the two major groups (1 & 2) previously described. While the overall profile of P. syzygii was sufficiently distinct from any other organism to support its position as a separate species, the presence (and absence) of certain fatty acids supported its membership of the *Pseudomonas* rRNA group 2 (which contains P. solanacearum and P.

cepacia) and the P. solanacearum/P. pickettii DNA homology sub-group.

The homology values obtained between P. syzygii isolates in DNA hybridisation studies consistently exceeded the minimum values for delineating species of bacteria. The homology values between P. syzygii and the other species studied showed P. syzygii to be more closely related to P. solanacearum (particularly biovar 1) than to any of the others. Measurements of the thermal stability of DNA hybrids showed P. syzygii to be most closely related to P. solanacearum, followed by P. cepacia and then P. fluorescens.

The G:C ratio of P. syzygii, determined to be 66-67% finally disproved the conjectured relationship with Xylella fastidiosa (G:C 52%). The G:C for P. solanacearum (also 66-67%) suggests but does not necessitate a relationship with P. syzygii.

1.1.10. Pseudomonas solanacearum

Bacterial wilt caused by P. solanacearum is one of the most important and widespread bacterial diseases of plants in the tropics, subtropics and warm temperate regions of the world (Kelman, 1953; Buddenhagen & Kelman, 1964; Persley, 1986b). The major economic hosts include potato, tomato, aubergine, pepper, ginger, peanut and banana.

P. solanacearum is also a significant pathogen on some economically important trees and shrubs, including teak, mulberry and olive (Hayward, 1985).

Table 1.1.1.

Cultural and Biochemical Characters Differentiating
Pseudomonas syzygii from Pseudomonas solanacearum

	<u>P.syzygii</u>	<u>P. solanacearum</u>
Motility	-	+/-
Flagella	-	+/-
NaCl Tolerance (w/v)	<0.5%	1.0%
Growth at 37°C	-	+
Growth on common media	poor or absent	good
Utilization of: ^a		
β-alanine, L-valine, Acetate, Lactate	0	100
L-Tryptophan, D-Trehalose, Glycerol	0	90
L-Leucine, D-Ribose	0	88
L-Threonine, D-Galactose	0	80
Tartrate	0	70
Sorbitol	0	75
Mannitol	0	50

Key:

^a = percentage of isolates tested giving positive results

1.1.11. *P. solanacearum* in Indonesia

The first report of bacterial wilt in Indonesia was in tobacco by Janse in 1892 (cited by Kelman, 1953) although he did not identify the causal bacterium.

Many other crops were subsequently found to be affected, including tomato, peanut, teak and cassava, and Kelman (1953) considered the importance of bacterial wilt in Indonesia to be second in the world after the USA, based on the number of available reports and economically important hosts affected by the disease.

Bacterial wilt has been reported from most areas of Indonesia including Sumatra, Java, Bali, Lombok and Sulawesi, and remains an economically important disease in many crops including potato, tomato, pepper, tobacco, cassava, peanut and ginger (Machmud, 1985).

1.1.12. Description of *P. solanacearum*

P. solanacearum is a Gram negative, motile (having a polar tuft of flagella), rod-shaped non-fluorescent pseudomonad (Palleroni & Doudoroff, 1971). The cells are non-pigmented but often form a diffusible brown pigment and accumulate poly- β -hydroxybutyrate as a cellular reserve material.

P. solanacearum belongs to the *Pseudomonas* rRNA homology group II (which also includes *P. caryophylli*, *P. cepacia* and *P. gladioli*). The GC content of the DNA ranges from 66.5-68 moles%.

P. solanacearum is a heterologous species with over 200 known hosts, specific strains showing varying degrees of host specificity. The most commonly accepted subdivisions of the species are the races of Buddenhagen and Kelman (1964) and the biovars of Hayward (1964).

The races are based on pathogenicity and can also be differentiated using infiltrated tobacco leaves. Three

racess have been described:

i). Race 1 infects most members of the Solanaceae and many other plants, and causes chlorosis (after 2 days) followed by wilting (7 - 8 days) in tobacco;

ii). Race 2 infects triploid banana and Heliconia spp. and produces a hypersensitive reaction after 12 - 24 hours in tobacco;

iii). Race 3 infects mainly potato and produces chlorosis (only) in tobacco after 2 - 8 days.

The four biovars of P. solanacearum are differentiated on the basis of the pattern of utilization of three hexose alcohols and three sugars.

There is much overlap between the two groupings and while race 3 isolates always belong to biovar 2, the reverse is not always the case and races 1 and 2 can contain all four biovars.

Apart from one strain of race 2 (causing Moko disease of bananas) which is insect transmitted (Buddenhagen & Elsasser, 1962), P. solanacearum is a soil borne disease agent.

Cook & Sequeira (1988) used restriction fragment length polymorphism (RFLP) analysis to assess the relatedness of P. solanacearum race and biovar isolates. They identified two major groups, group I included strains of race 1, biovars 3 and 4; and group II included strains of race 1 biovar 1, and races 2 and 3. Race 2 strains formed three distinct groups, each group correlating with geographical origin. SDS-PAGE analysis of P. solanacearum biovars 1, 2 and 3, revealed similar patterns in all isolates, except for the absence of a major 36 kDa protein in biovar 2. There was no relationship between pattern and host or geographical origin of the isolates (Dristig & Dianese, 1988). The role of P. solanacearum as a plant pathogen is discussed further in section 1.2.

1.1.13. The Banana Blood Disease Bacterium

Blood disease of bananas is confined to the island of Sulawesi (formerly Celebes) in Indonesia and some parts of Java. It is distinct from Moko disease (not present in Indonesia) and produces a white to reddish-brown "blood-like" exudation from cut fruits (Tjahyono & Eden-Green, 1988).

The disease is caused by a Gram negative bacterium that is non-pathogenic to solanaceous hosts.

Bacteriological characterization of the organism suggested that the existing taxonomic designation of Xanthomonas campestris pv. celebensis was erroneous, and that a previous name, Pseudomonas celebensis be reinstated (Eden-Green et al., 1988). As this name has not been accepted at the time of preparation of this thesis, the organism will be referred to as the Banana blood disease bacterium (BDB).

BDB isolates were found to react with antisera to both P. syzygii and P. solanacearum, suggesting a close relationship between the three species (Dr S.J. Eden-Green, personal communication).

1.2. BACTERIAL DISEASES OF PLANTS

(With Special Reference to Vascular Diseases)

1.2.1. Plant Pathogenic Bacteria

The majority of bacteria causing disease in plants are Gram-negative, straight or slightly curved rods with rigid cell walls, with aerobic or facultative anaerobic metabolism and belong to the genera Pseudomonas, Xanthomonas, Erwinia, Agrobacterium (Bradbury, 1986), and Xylella (Wells et al. 1987).

Some Gram-positive species of Corynebacterium (Vidaver, 1982), Nocardia and Streptomyces also cause plant disease, and can be distinguished from Gram-negative bacteria on the basis of Gram-staining, and from each other by their morphology (i.e. Corynebacteria have coryneform (pleomorphic cells) morphology, whereas Streptomyces and Nocardia produce branched mycelia) (Bradbury, 1986).

Most important phytopathogenic bacteria formerly classified as Corynebacterium have now been placed in the genus Clavibacter (Davis et al. 1984).

Bacteria-like organisms (BLO) such as the citrus-greening organism (Garnier et al. 1984) and the cell wall-less mycoplasma-like organisms (MLO) (Bove, 1984) (which includes spiroplasmas) have also been implicated as causal agents of plant disease.

With the exception of Xylella spp., P.syzygii, BLOs and MLOs, plant pathogenic bacteria can be grown on relatively simple media although they are generally outgrown by saprophytes on primary isolation.

Full descriptions of the phytopathogenic bacteria and details of isolation, culturing, and identification can be found in the texts of Bradbury (1986), Lelliott and Stead (1987), and Fahy and Persley (1983).

The Gram-negative genera can be differentiated

primarily by flagella arrangement and carbohydrate metabolism (see Table 1.2.1).

Several of the genera are subdivided into groups. For example, Erwinia consists of three groups; Amylovora (slow growing, non-pectolytic), Herbicola (faster-growing, non-pectolytic, yellow pigmented) and Carotovora (fast-growing, pectolytic).

Pseudomonas can be roughly divided into fluorescent (i.e. produce a water soluble, greenish fluorescent pigment on King's Medium B) and non-fluorescent groups. Fluorescent Pseudomonads (e.g. P. syringae) can be further subdivided into five groups based on the LOPAT test scheme of Lelliott et al. (1966, cited by Lelliott and Stead (1987)). Most non-fluorescent Pseudomonads (e.g. P. solanacearum) produce poly- β -hydroxybutyrate storage granules.

Many species are very heterogenous consisting of many pathovars differentiated largely on phytopathogenicity characteristics, e.g. Xanthomonas campestris includes approximately 120 pathovars.

Others such as P. solanacearum are subject to overlapping division based on host range (races) or biochemical characteristics (biovars).

Heterogeneity of a species is generally reflected in its host range (i.e. pathogens with narrow host ranges are usually homogenous and defined within narrow taxonomic limits and vice versa).

1.2.2. Distribution

Most bacterial diseases of plants are restricted to tropical, sub-tropical, or warm-temperate areas. Only a few are of importance in temperate areas such as the U.K., e.g. fireblight of rosaceous plants (Erwinia amylovora), and potato blackleg (Erwinia carotovora subsp. atroseptica).

Table 1.2.1.

Differentiation of Gram-negative Bacterial Plant Pathogens

<u>Genus</u>	<u>Flagella</u>	<u>Metabolism</u>
<u>Erwinia</u>	peritrichous	facultatively anaerobic
<u>Xanthomonas</u>	single polar	strictly aerobic
<u>Pseudomonas</u>	1 or more polar*	strictly aerobic
<u>Agrobacterium</u>	sub-polar or peritrichous	strictly aerobic
<u>Xylella</u>	none	strictly aerobic

* P. syzygii is aflagellate.

1.2.3. Symptoms (Lelliott & Stead, 1987; Agrios, 1988)

Symptoms of bacterial infection can include most disease symptoms found in plants, either singly or in combination. The most important are:

1). Hyperplasias; which are diseases in which abnormal growth has occurred and include fasciations, root and shoot proliferation, galls and hyperplastic cankers. All appear to arise as a result of impaired hormonal or growth regulation. The most studied example is Agrobacterium tumefaciens which causes crown gall as result of the incorporation of bacterial plasmid borne genes into the host genera (Nester & Kosuge, 1981).

2). Bacterial Spots and Blights; the most common type of bacterial diseases of plants appearing as spots of various sizes on leaves, stems, blossoms and fruits. Some bacterial diseases appear as continuous, rapidly advancing

necroses, and are termed blights.

Following entry through natural openings (e.g. stomata) or wounds, the bacteria multiply and spread in the intracellular spaces. Colonized tissue usually dies fairly rapidly and becomes necrotic. Depending on the pathogen involved and the physiological state of the host, the spread of the pathogen in tissue is either soon stopped, forming spots, or continues to form large necrotic areas (necroses).

Most bacterial spots and blights are caused by members of the genera Pseudomonas and Xanthomonas, particularly P. syringae and X. campestris (Hirano & Upper, 1983).

3). Bacterial cankers; the symptoms of canker on stems, branches and twigs (either sunken and soft, splits in the stem, necrotic areas or scabby excrescences on the surface of the tissue) are only part of the disease syndrome. Direct symptoms on fruits, leaves, buds and blossoms may be at least as important in the overall effect of the disease.

Bacteria enter the parenchyma tissues of stems beneath the bark or epidermis via wounds, lenticels or leaf scars. The most important bacterial cankers are those of stone fruit and pome trees, caused by P. syringae pv. syringae and P. syringae pv. morsprunorum (Crosse, 1966) and citrus canker, caused by X. campestris pv. citri (Kuhara, 1978 and Stall & Seymour, 1983).

4). Bacterial Soft Rots; occur most commonly on vegetables that have fleshy storage tissues (e.g. potatoes and onions) or succulent stem, stalk or leaves (e.g. cabbage, spinach). They are a worldwide problem, causing serious losses in the field, in transit, and particularly in storage.

The symptoms start on fruits and other fleshy organs as a small rapidly expanding water-soaked lesion. The affected area becomes soft and mushy and surface discolouration or wrinkling may occur. Complete tissue maceration may occur within 3 - 5 days.

Entry of bacteria is usually via wounds but uninjured tissues may become infected during storage.

The major soft-rotting bacteria are within the genera Erwinia and Pseudomonas e.g. E. carotovora is a causal agent of numerous soft rots (Perombelan & Kelman, 1980).

5). Bacterial Vascular Wilts; vascular wilts diseases are distinct from those that produce local lesions in that the infection may become systematic. Because a wilt pathogen resides principally in the xylem vessels (Pegg, 1985), its propagules and the products of its action or interaction with the host, may be moved throughout the stem and leaves with the transpiration stream (Dimond, 1970). Bacteria from several genera can cause vascular wilts (see Table 1.2.2.).

Vascular wilts are also caused by three genera of fungi, Ceratocystis (Strobel & Lander, 1981), Verticillium (Pegg, 1974) and Fusarium (Armstrong & Armstrong, 1975).

The presence and activity (e.g. movement, multiplication) of a pathogen within the xylem vessels of the host plant interferes with the translocation of water and nutrients which results in the drooping, wilting and death of the aboveground parts of the plant.

Ultimately, most wilt pathogens cause plants to wilt by increasing the resistance to water flow through the xylem (Beckman, 1987 as cited by Van Alfen, 1989).

While vascular fungi usually remain within the vascular tissues until the death of the plant, bacteria dissolve or rupture vessels, resulting in their spread into adjacent parenchyma tissues (Pine et al. 1955 and Nelson & Dickey, 1970). Killing of parenchyma cells produces cavities full of bacteria, gums and cellular debris.

Cutting of infected stems may result in the exudation of bacterial ooze.

Browning and/or hyperplasia of xylem parenchyma may occur, the latter resulting in the crushing of xylem vessels (Dimond, 1970).

Symptoms of bacterial wilt on solanaceous crops caused

by P. solanacearum appear as a rather sudden wilt with young plants dying rapidly. The full range of external symptoms may include wilting, yellowing, marginal necrosis of leaf margins, leaf epinasty, adventitious roots and stunting, while internal examination may reveal vascular discolouration, tyloses, vessel collapse, parenchyma proliferation, dissolution of pectic substances in middle lamella and degradation of cellulose in cell walls (Buddenhagen & Kelman, 1964).

6). Diseases Caused by Fastidious Xylem-Limited Bacteria XLB

Fastidious XLB were first associated with plant disease with the discovery of the bacterial etiology of Pierce's disease of grapevine by Davis et al. (1978).

Similar bacteria have also been associated with several other diseases including phoney disease of peach (Wells et al. 1983), stunting of ragweed (Timmer et al. 1983), leaf scorch of almond (Mircetich et al. 1976), sycamore (Sherald et al. 1983) and plum (Raju et al. 1982).

These bacteria are now all included in the species Xylella fastidiosa and are thought to be most closely related to the Xanthomonads (Wells et al. 1987).

Symptoms of disease caused by X. fastidiosa include leaf burning or scorching (the most characteristic symptom), slow decline, stunting, reduction in fruit yields, chlorosis, root system decline and sometimes, but not always, death (Raju & Wells, 1986). Wilting does not always occur (Hopkins, 1977).

All diseases caused by X. fastidiosa are vector transmitted. The causal agent of Sumatra Disease of cloves, P. syzygii is also an insect-transmitted XLB, but is completely unrelated to X. fastidiosa (Roberts et al. 1990).

Table 1.2.2
Vascular Wilts Caused by Bacteria

Pathogen/host interactions for which vascular wilting is a major symptom are summarized in table 1.2.2.

<u>GENUS</u>	<u>SPECIES</u>	<u>DISEASE</u>	<u>REFERENCE</u>
<u>Clavibacter</u> (<u>Corynebacterium</u>)	<u>C. michiganense</u> subsp. <u>insidiosum</u>	bacterial wilt of alfalfa	Hayward & Waterston (1964)
	<u>C. flaccumfaciens</u>	bacterial wilt of bean	Hayward & Waterston (1965)
	<u>C. michiganense</u> subsp. <u>sepedonicum</u>	ring rot of potato	De Boer & Slack (1984)
	<u>C. michiganense</u> subsp. <u>michiganense</u>	bacterial canker & wilt of tomato	Grogan & Kendrick (1953)
<u>Erwinia</u> N G	<u>E. tracheiphila</u>	bacterial wilt of cucurbits	Nuttall & Jasmin (1958)
	<u>E. stewartii</u>	Stewart's wilt of corn	Bradbury (1967)
	<u>E. amylovora</u>	fire blight of pome fruits	Eden-Green & Billing (1974) Schroth et al. (1974)
<u>Pseudomonas</u>	<u>P. solanacearum</u>	(southern) bacterial wilt of solanaceous crops	Buddenhagen & Kelman (1964)
		Moko disease of bacteria	Sequeira (1958)
	<u>P. caryophylli</u>	bacterial wilt of carnation	Bradbury (1973a)
<u>Xanthomonas</u>	<u>X. campestris</u> p.v. <u>campestris</u>	black rot of crucifers	Williams (1980)
	<u>X. vascularum</u>	gumming disease of sugarcane	Bradbury (1973b)

1.2.4. Transmission and Pathogen Entry

Plant pathogenic bacteria are unable to penetrate intact plant surfaces, entry being via natural openings such as stomata (Panopoulos & Schroth, 1974), hydathodes (Staub & Williams, 1972), lenticels (Lund, 1979) and leaf scars (Feliciano & Daines, 1970), or wounds (e.g. caused by wind, hail, insects and cultural practices) (Billing, 1982).

Young tissue is more susceptible than mature tissue and water-soaking greatly increases chances of infection as it allows free passage of bacteria from outer to inner surfaces. Plants are particularly vulnerable when damage and water congestion coincide (e.g. storms), torn leaves with their vascular system exposed being particularly vulnerable to infection (Crosse et al. 1972; Daft & Leben, 1972).

The majority of plant pathogenic prokaryotes do not require an active vector to infect plants. In some cases, e.g. E. amylovora, insect vectors may be important but are not indispensable in dispersing to new hosts (Schroth et al. 1974). For diseases caused by fastidious xylem-limited bacteria (XLB) belonging to X. fastidiosa (Raju & Wells, 1986) and mycoplasmas (Daniels et al. 1982), insect vectors are necessary.

The vectors of X. fastidiosa are xylem-feeding sharpshooter leafhoppers (Cicadellidae) and spittlebugs or froghoppers (Cercopidae of the order Homoptera) (Purcell, 1979, as cited by Davis et al. 1981).

The relationship of X. fastidiosa with its vectors differs markedly from that of other prokaryotes (Purcell & Finlay, 1979), with transmission of bacteria being possible within two hours or less after acquisition from infected plants, suggesting a non-circulative association. Purcell et al. (1979) and Brulansky et al. (1983) concluded that the bacteria colonize the foregut area of the vector and are transmitted by expulsion from the foregut during feeding.

Infected planting material is the major means of dispersal of P. solanacearum from place to place and season to season, particularly in vegetative seed pieces of crops such as potato and ginger (Persley, 1986a). Latent infection of potato seed pieces has been demonstrated, with strains differing in their ability to establish latent infection in resistant and susceptible potato clones (Gampi & Sequeira, 1980 and Gampi et al. 1980).

Few studies have considered the possibility of dissemination of P. solanacearum on true seed (Devi & Menon, 1980 as cited by Persley, 1986a).

Artificially infested seed of tomato and capsicum was shown to act as a source of inoculum for the emerging cotyledons (Moffett et al. 1981).

Such a mode of transmission was thought to be more important than recognised (Persley, 1986a), particularly as tomato seed can be infected by other xylem-inhabiting bacteria, i.e. C. michiganense (Stapp, 1961 as cited by Buddenhagen & Kelman, 1964).

Localised spread can also occur by root-to-root spread in tomato and tobacco (Kelman & Sequeira, 1965). P. solanacearum can also enter roots at cracks where secondary roots emerge (Schmidt, 1978) and ingress into tobacco was shown to be facilitated by the activities of the root-knot nematode (Johnson & Powell, 1969).

There is disagreement in the literature on both the survival of P. solanacearum in soil and the significance of weed hosts in increasing the inoculum potential of soils (Hayward, 1986).

The P. solanacearum Race 2 SFR strain which causes Moko disease of bananas in Central and South America is transmitted by a variety of insects including bees, wasps and fruit flies (Buddenhagen & Elsasser, 1962 and French & Sequeira, 1970). The bacteria are mechanically carried from ooze from diseased banana inflorescences, invasion occurring not through flowers but at open xylem vessels exposed when bracts and male flowers dehisce naturally.

1.2.5. Xylem and the Movement of Bacterial Vascular Pathogens

The xylem is a continuous tissue throughout the plant, and has three primary functions: transport of water and nutrients; storage and structural support (Esau, 1965, as cited by Davis et al. 1981). Xylem consists of several types of cells, the dead and empty water-conducting elements (tracheids and vessels), the fibres and sclereids which add support, and living parenchyma cells of various function (Vines & Rees, 1972).

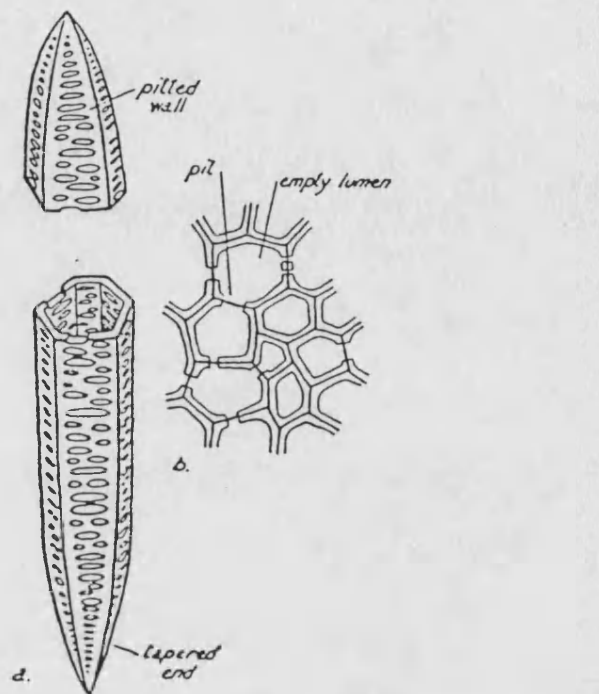
Tracheids are elongated with finely tapering ends, with a heavily lignified and pitted secondary cell wall and no protoplast. All tracheary elements are connected by paired pits in the intervening walls (see Figure 1.2.1).

Mature vessels are long, open, pipe-like units, composed of a number of vessel members whose adjoining ends have open perforation plates. Lateral interconnection may also be achieved through paired pits. Vessels are of finite length but may extend up to several metres (Esau, 1965 as cited by Davis et al. 1981) (Figure 1.2.1.).

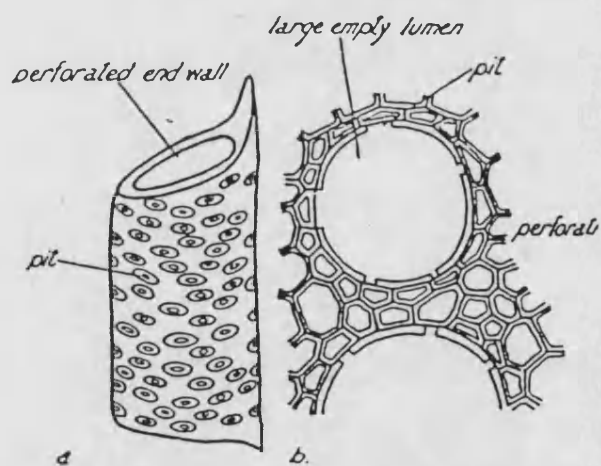
Xylem parenchyma cells form the medullary rays and may also form longitudinal strands through the xylem, and may contain starch, tannins and other substances (Vines & Rees, 1972). Parenchyma cells may develop ingrowths (tyloses) into the adjacent tracheids or vessels through the paired pits. Tyloses are common in older wood or injured tissue.

The xylem constitutes a unique physical and nutritional environment. The ascending flow of sap from the roots fluctuates in response to transpiration of water by the plant and the flow may reverse under conditions of high atmospheric humidity and low soil moisture (Bollard, 1960). It is likely that sap flow is important in bacterial dissemination within the plant, particularly in the case of the non-motile Xylella

Figure 1.2.1
The Structure of Xylem Tracheids and Vessels
 (From Vines & Rees, 1972)



Xylem. (a) and (b) Generalized tracheid whole and in T.S.



Xylem. (a) Single vessel element (whole). (b) T.S. vessel.

species and P. syzygii (Davis et al. 1981). Vascular pathogens obtain nutrients from the xylem-fluid and also probably by diffusion from adjacent living cells.

The composition and concentration of solutes in xylem fluid varies with plant species, age, health, nutritional state, location within the plant, time of day and seasonal cycle. The xylem-fluid is a qualitatively rich but quantitatively dilute nutritional environment, containing 1-20 mg/ μ l solids in contrast to phloem sap which contains 50-300 mg/ μ l solids (Pate, 1976). These solids contain a vast array of soluble compounds that could serve as bacterial nutrients.

The oxygen supply in xylem fluid is less than in the external environment and has been estimated at 0.8 ppm (at 20°C) in tomato plants which is about one tenth of saturation (Dimond, 1962).

Bacterial pathogens may enter tracheids and vessel elements either directly (when ingress occurs through wounds which directly injure vascular tissue or via xylem feeding insects) (Nelson & Dickey, 1970), or after initial multiplication in adjacent tissues either after entry via wounds or natural openings such as hydathodes (Staub & Williams, 1972).

The bacteria are usually confined initially to the xylem tracheids and vessels until they fill the lumens of the invaded vessels (Nelson & Dickey, 1970). After filling the lumen. the bacteria may move into the pit apertures and cavities.

Pathogen spread is both upward and downward through the xylem from the initial point of entry (Nelson & Dickey, 1970).

Xylem-limited bacteria move from one tracheary element to another by breaching pit membranes; however, pit membranes probably limit lateral spread as bacteria-free tracheary elements are often observed adjacent to those packed with bacteria (Davis et al. 1981). The open perforation plates of vessels allow relatively unimpeded

longitudinal spread (Wolf & Nelson, 1969).

There is strong evidence that the mechanism of pathogenicity of X. fastidiosa is the production of relatively mild but prolonged water stress due to vascular occlusions (i.e. bacterial aggregates, host gums and tyloses) (Goodwin et al. 1988 and Hopkins, 1989).

Once established in the xylem vessel elements vascular bacterial pathogens move out into the surrounding tissues following rupture of the vessel element due to internal bacterial pressure or cell wall dissolution (Nelson & Dickey, 1970). This is followed by the formation of the bacterial pockets.

1.2.6. Pathogenicity and Virulence Factors

For maximum pathogenic potential it is important for invading bacteria to colonize tissue rapidly and to reach high population levels before plant tissue maturation or other plant responses limit bacterial growth (Billing, 1987).

Ideally, pathogen products with a major role in pathogenicity (ability to produce disease) should be distinguished from those affecting virulence (ability to increase disease severity) but this is not always easy (Billing, 1987).

A wide variety of molecules may be involved in the production of plant disease:

a). Polysaccharides; There is considerable data supporting an essential role of capsular polysaccharides (exopolysaccharides = EPS) in phytopathogenicity of such bacteria as E. amylovora (Billing, 1960 and Bennett & Billing, 1980), P. solanacearum (Kelman, 1954), other Pseudomonads and Xanthomonads (Goto, 1972). The two principle observations supporting such a role are altered virulence of capsular mutants and pathological responses induced by fractionated capsular polymers.

In the case of P. solanacearum these findings have recently been challenged by Xu et al. (1990) who isolated a transposon (Tn5) induced mutant unable to produce EPS in planta but which could cause severe wilting. In addition, avirulent mutants that still produce EPS have also been isolated (Xu et al., 1988).

Xu et al. (1990) suggested that the conflicting evidence may be due to the fact that the strongest evidence linking EPS to pathogenicity has been obtained from work done with the spontaneous avirulent mutant B1 (Kelman, 1954). The shift to the B1 phenotype is highly pleiotropic and involves changes not only in EPS production but also in lipopolysaccharide (LPS) composition, induction of the hypersensitive response, piliation and indole acetic acid (IAA) production amongst others (Buddenhagen & Kelman, 1964; Duvick & Sequeira, 1984; Hendrick & Sequeira, 1984; Whatley et al. 1980).

On the basis that very small amounts of LPS could cause plant cuttings to wilt, it has been suggested that EPS can act as a toxin (Ries & Strobel, 1972). However, it has been shown that the vascular dysfunction caused by EPS is the result of plugging of the pit membrane pores, with only picogram quantities being required (Van Alfen et al. 1983).

Other functions of capsular EPS that have been suggested include survival outside the host, chelation of metals, nutrient uptake and water retention (possibly causing suppression of the hypersensitive reaction (see 1.3.8)).

Capsules may also mask sites on the outer membrane complementary to plant components thus preventing bacterial attachment to host cells. Results with P. solanacearum and other phytopathogenic bacteria (Sequeira, 1979 as cited by Chatterjee & Vidaver, 1986). Smith & Mansfield (1982) and Fett & Jones, (1983) suggest that non-encapsulated cells attach to host cells, elicit a defence reaction and are inactivated or eliminated, while capsulated cells generally

remain free and multiply.

b). Cell Wall Degrading Enzymes; pectolytic enzymes are produced by soft-rot bacteria (e.g. E. carotovora) (Stack et al. 1980) and break down the pectic substances of the middle lamella and of the cell wall which, in conjunction with cellulolytic enzymes which cause partial breakdown and softening of the cellulose of the cell walls, results in diffusion of water from protoplasts into the intracellular spaces followed by plasmolysis, collapse and death of the cells (Agrios, 1988).

P. solanacearum produces several types of cell wall degrading enzymes (Husain & Kelman, 1958), but it is unclear exactly how and to what extent they function in disease. Ultrastructural and light microscopy studies have shown that P. solanacearum degrades plant cell wall material (Husain & Kelman, 1958; Kelman & Cowling, 1965 and Wallis & Truter, 1978) suggesting that cell wall degrading enzymes may be involved in the progression of the pathogen through plant tissues (Husain & Kelman, 1958 and Kelman & Cowling, 1965).

Husain & Kelman (1958) concluded that cell wall degrading enzymes may also play a minor role in inducing wilt due to their extensive degradation of the vascular system.

Further evidence for the role of cell wall degrading enzymes in pathogenesis comes from the work of Roberts et al. (1988), in which a strain of P. solanacearum deficient in B-1, 4-endoglucanase (which leaves soluble cellulose, releasing cellobiose) was shown to be less virulent on tomato plants than the wild type parent. In addition, Schell et al. (1988) isolated a polygalacturonase deficient mutant that was reduced in virulence on tomato plants. They suggested that the enzymatic action of both the pglA and egl products may facilitate penetration of cortical tissue at wound sites or aid in the progression of bacteria through the plants. The enzymes do not function in nutrition as P. solanacearum cannot utilize their products

(cellobiose and polygalacturonic acid) as sole carbon and energy sources.

Roberts et al. (1988) suggested that pathogenicity is a complex polygenic phenotype involving genes of varied importance.

c). Plant Growth Regulators: P. solanacearum produces IAA (indole acetic acid) in culture, which has been linked in vivo to the external symptoms of adventitious root formation and leaf epinasty and the internal symptoms of parenchyma proliferation, vessel collapse and tylose formation (Buddenhagen & Kelman, 1964).

Infected banana and tobacco plants contain elevated levels of IAA (Sequeira & Kelman, 1962), but most IAA present in early infection is host derived (Sequeira, 1965), however IAA synthesis in the host is essentially inoperative in advanced stages of pathogenesis and therefore IAA present at this stage may be pathogen derived.

Ethylene is also produced by P. solanacearum in vitro and in addition to IAA, contributes to leaf epinasty as high ethylene levels occur in diseased, prematurely yellowing banana fruit (Freebairn & Buddenhagen, 1964). Again, however, it was shown that the host was responsible for ethylene production (Pegg & Cronshaw, 1976).

The pathogen may still be ultimately responsible for the increased levels of the substances as it induces changes in the shikimic acid pathway in the host plant, leading to tryptophan accumulation, which would account for increases in IAA and ethylene production by the host plant (Billing, 1987).

d). Toxins: Phytotoxin production appears to be limited to the fluorescent pseudomonads and in particular the pathovars of P. syringae (Gross & Cody, 1985). This group of pathogens are generally host specific and invade parenchyma tissues, eliciting chlorosis and/or necrosis symptoms that in many cases can be attributed to excretion of one or more phytotoxins. The phytotoxins are low

molecular weight substances, in many cases exhibiting a peptide or amino acid nature (Mitchell, 1981 as cited by Gross & Cody, 1985).

Phytotoxins have the basic attributes of disturbing the metabolism of the host and enhancing access to essential nutrients (Gross & Cody, 1985). Some of the better studied toxins include, phaseolotoxins, coronatine, syringomycin and tabtoxin.

Phytotoxins are considered to be virulence rather than pathogenicity factors as some tox⁻ strains can invade and multiply in host plants tissue and produce local lesions, the toxin appears necessary for full invasive capacity and typical symptom expression (Billing, 1987).

1.2.7. Genetics of Pathogenicity Determinants

The ability of P. solanacearum to induce a disease on a compatible host and ability to induce HR on an incompatible host share a certain number of common functions encoded by genes designated hrp (hypersensitive, response and pathogenicity) (Boucher et al. 1985), which may be clustered (Boucher et al. 1987) on a megaplasmid (Boucher et al. 1986).

Sequences homologous to the hrp cluster are also found in X. campestris but not in closely related non-phytopathogens, suggesting that the hrp genes encode for functions specific to phytopathogenic bacteria belonging to different taxa and that basic pathogenicity functions may be shared among bacteria inducing entirely different types of disease (Boucher et al. 1987).

1.2.8. Plant Defence Mechanisms

The methods by which plants defend themselves against pathogens can be divided into two main categories:

- 1). Structural characteristics that act as physical barriers and inhibit the pathogen from gaining entry;

2). Biochemical reactions that take place in the cells and tissues of the plant to produce substances that are either toxic to the pathogen or create conditions that inhibit growth of the pathogen.

Structural defences may either be pre-existing or formed in response to infection.

Pre-existing defences include the amount and quality of wax and cuticle that cover the epidermal cells (Martin, 1964); the structure of the epidermal cells; the size, location and shapes of stomata and lenticels, and the presence on the plant of tissues made of thick-walled cells that hinder the advance of the pathogen (Agrios, 1988).

The major defence structures formed in response to bacterial pathogens include cork layers (formed ahead of the pathogen, they inhibit movement and deprive it of its nutrients), abscission layers (whereby the invaded area of the leaf is cut off and removed), tyloses (which can block xylem vessels) and gums (which enclose and isolate the pathogen) (Agrios, 1988).

The specificity of many plant microbe interactions implies some mechanism of recognition, one of which may be the exchange of molecular signals between host and microbe (Halverson & Stacey, 1986).

Such signal molecules can have either a positive or a negative effect on the interaction. A microbe's recognition of the host's cell surface or a component of it could induce the physiological responses necessary for the interaction. Conversely, recognition of a microbe by a host may induce the expression of genes necessary to defend against, or enhance, the interaction. The exchange of signal molecules (which may be DNA, RNA, protein, lipid or polysaccharides) appears to be necessary for many plant-microbe interactions (Halverson & Stacey, 1986).

Recognition of a potential pathogen by the host can elicit a number of defence mechanisms, including antimicrobial phytoalexins, the hypersensitive response (HR), and cell wall modification. Conversely, recognition

of a host by the pathogen can induce the development of parasitic forms of the pathogen.

Phytoalexins are a chemically heterogeneous group of low molecular weight compounds with antimicrobial properties (Lyon & Wood, 1975) and are not present in healthy plant tissues, but appear at the site of an infection (Bell et al. 1984, and Mansfield et al. 1974).

Phytoalexin synthesis can be induced by a number of substances (elicitors) (Darvill & Albersheim, 1984), including endopolygalacturonase from bacterial cell walls (Davis et al. 1984), which acts by releasing an endogenous elicitor from the plant cell wall.

The importance of phytoalexins in defence against bacteria is not clear (Billing, 1987), their accumulation being mostly associated with HR, which is a response to exposure to an incompatible pathogen (Kelman & Sequeira, 1972). HR involves rapid electrolyte leakage from, and loss of turgor of, plant cells, followed by desiccation and necrosis and the accumulation of inhibiting substances including phytoalexins. Non-pathogens produce little damage, whereas compatible pathogens produce delayed electrolyte leakage, followed by water soaking and progressive infection.

The concentration of hydroxyproline rich glycoproteins in the regions of the plant cell wall affected by disease increases when infection occurs. This increase correlates with a decreased susceptibility, and reduced levels correlate with an increased susceptibility, to infection (Hammerschmidt et al. 1984).

The avirulent P. solanacearum strain B1 attaches to suspension cultured tobacco cells and tobacco leaf cell walls and is strongly agglutinated by a hydroxyproline rich glycoprotein extracted from potato tubers, whereas the virulent strain K60 binds poorly and is weakly agglutinated (Duvick & Sequeira, 1984; Sequeira & Graham, 1977).

Agglutination of avirulent P. solanacearum strains lacking EPS is prevented by the addition of purified EPS

(Sequeira & Graham, 1977). LPS has been suggested as being the receptor for the glycoprotein (Sequeira & Graham, 1977). Duvick and Sequeira (1984) suggested that binding was insufficient to trigger a host response, and that specificity of the interaction results from events occurring after attachment.

1.2.9. Control

Bacterial diseases of plants have in general proved difficult to control, this is partly due to low investment in research due to their relatively low importance in comparison to fungal and viral diseases (Billing, 1977).

In addition there are many intrinsic factors of bacterial disease which make control difficult. Many bacterial pathogens spend little time on plant surfaces and may not be easily controlled by surface sprays; they tend to grow more rapidly than fungal pathogens and are not susceptible to the majority of antifungal agents; and the EPS produced by many bacterial pathogens may protect them from some antibacterial agents (Billing, 1987).

Present approaches to control include, breeding for resistance, cultural measures and crop hygiene, the use of chemical agents and biological control.

Some success has been achieved in the development of resistant crops, e.g. in potato (Sequeira & Rowe, 1969 and Thurston, 1976) and tomato (Mew & Ho, 1976) to P. solanacearum. Varieties bred for resistance in one area frequently do not sustain their resistance when transferred elsewhere (Rau et al. 1975 and Sonoda & Augustine, 1978).

Grafting onto resistant rootstocks (e.g. tomato scions on to P. solanacearum resistant aubergine) has also been successful (Thurston, 1976).

Preventative cultural and hygiene measures include; avoiding the use of susceptible crops in climatic areas where disease risks are high; the use of certified and/or

quarantined planting material; avoiding tissue damage (especially during rain) from wind, insects, machinery, tools etc., and the transfer of infection on tools; the destruction of all infected plants and debris; and rotation where soil infestation is a problem (Billing, 1987). Control of the vector borne diseases may be achieved through eradication of the vector.

Intercropping may also reduce disease incidence. Autrique & Potts (1987) demonstrated a marked reduction of infection of potato by intercropping with maize or haricot beans, the increased distances between individual potato plants and the presence of other plant roots systems between them, being thought to be the important factors.

Chemical agents have seen limited application in the control of bacterial plant diseases. Antibiotics have been applied to the treatment of Sumatra Disease of Cloves (Hunt et al. 1985) and fire blight (Aldwinckle & Beer, 1979) but are of limited use due to potential phytotoxicity, the requirement for frequent applications and the possible contamination of fruits intended for human consumption. Copper based compounds e.g. Bordeaux mixture have also been used with fire blight (Aldwinckle & Beer, 1979).

Control of crown gall (caused by Agrobacterium tumefaciens) has been achieved by using A. radiobacter (Kerr, 1980) which produces a bacteriocin (an antibiotic like compound with bactericidal specificity restricted to bacterial strains closely related to the producer (Vidaver, 1983)). Bacteriocin-producing avirulent strains of P. solanacearum protected tomato plants against infection by virulent strain (Tsai et al. 1985).

1.3. MONOCLONAL ANTIBODIES

Antibodies (Abs) belong to a heterogenous group of glycoproteins known as immunoglobulins (Igs) that are found in all vertebrate species. They can recognise and bind a wide range of foreign substances (antigens (Ags)), and subsequently trigger further defence mechanisms at the molecular or cellular level (Roitt, 1988; Male et al., 1987).

The unique diversity and specificity of Abs, first recognised by Landsteiner in 1936, makes them ideally suited for use in any area of research or applied science where specific recognition is required.

Abs have been used as tools for many years, but the serious exploitation of their properties was prevented by the lack of suitably sensitive analytical methods and the heterogeneity of the immune response, which precludes the production of antisera of the purity and reproducibility required by a true chemical reagent.

The ability to produce a homogenous population of antibody molecules of predetermined specificity (monoclonal antibody (mAb)) in virtually unlimited quantities is a relatively recent development. MAb production by the hybridoma technique was first described by Kohler & Milstein (1975) and its importance was recognised by the award of the Nobel Prize in Physiology and Medicine in 1984.

Several important earlier discoveries which laid the basis for the development of the hybridoma technique include; the proof of the clonal selection theory (postulating that each lymphocyte had a unique receptor specificity, and was thus precommitted to making only one Ab after appropriate stimulation (Burnet, 1957)) by Nossal & Lederberg (1958), the development of cell fusion techniques (Okada, 1962; Littlefield, 1964), the artificial induction of plasmacytomas (Potter & Boyce, 1962) and their adaptation to in vitro culture (Horibata & Harris, 1970), and the fusion of plasma cell lines with the retention of Ab production (Cotton & Milstein, 1973).

1.3.1. Antibody Structure

All Ab molecules have the same basic structure consisting of two identical, glycosylated heavy polypeptide chains (H chains) (of 50 - 75 kDa) and two identical light chains (L chains) (of approximately 25 kDa) (Porter, 1967; Kabat, 1976 (as cited by Wall & Kuehl, 1983)), which are cross-linked and stabilized by interchain disulphide bonds and secondary interactions (see Figure 1.3.1).

Igs are grouped into five main classes (IgM, IgD, IgG, IgE, and IgA), possessing distinctive structural and biological properties. IgG is further divided into four subclasses in humans (IgG1, IgG2, IgG3, & Ig4), mice (IgG1, IgG2a, IgG2b, & IgG3), and rats (IgG1, IgG2a, IgG2b, & IgG2c). Class and subclass are determined by the heavy chains. There are two light chain types, kappa (K) and lambda (L). Individual Ig molecules may possess either light chain type, but never both.

H and L chains are made up of a series of homology units (of about 110 amino-acids) which are stabilized by intrachain disulphide bonds. Each homology unit is folded into a domain (Edelman & Gall, 1969), which is a compact globular structure with a characteristic beta-pleated sheet protein structure. H chains of IgG consist of four domains (V_H , C_H1 , C_H2 , and C_H3), whereas L chains have two domains (V_L and C_L). All domains except C_H2 form contacting pairs linked by non-covalent bonds (Huber et al., 1976; Deisenhofer et al., 1976).

The amino-acid sequence of the N-terminal domains (the variable (V) domains) shows considerable variability between different Ig molecules, most of which is concentrated in three segments (the hypervariable regions (Wu and Kabat, 1970)). Association of the paired V_H and V_L domains brings the hypervariable regions together to form

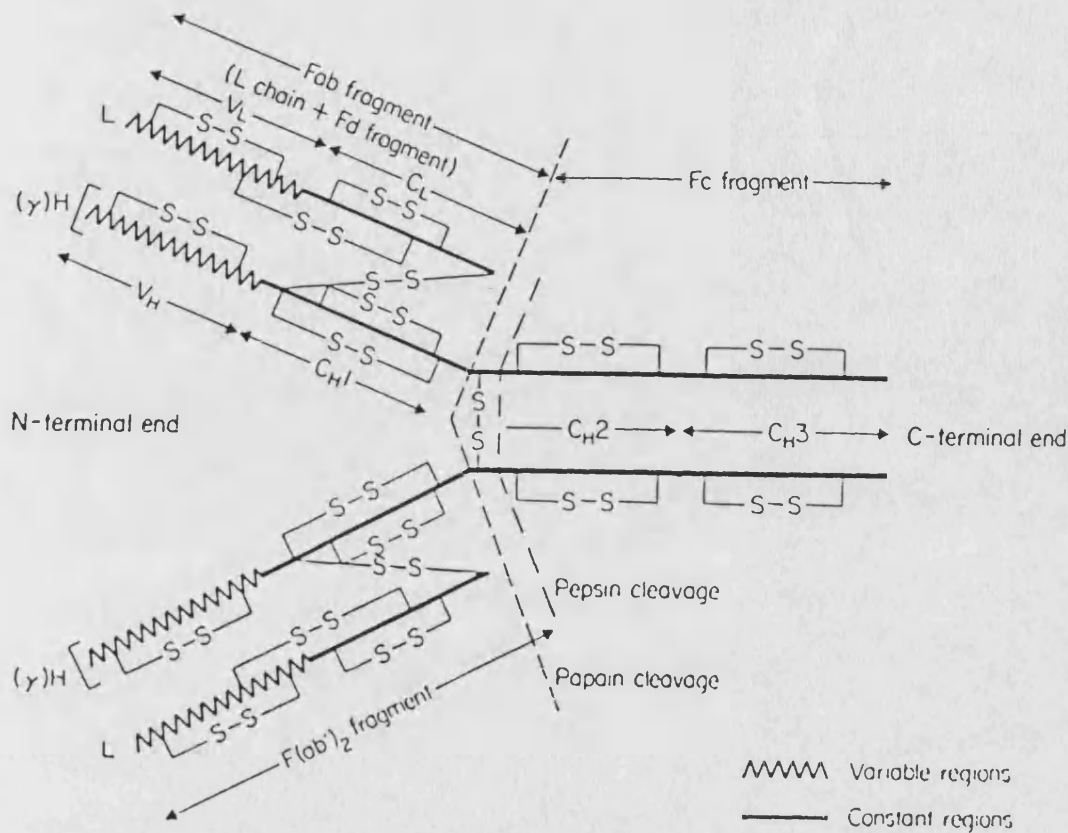


Figure 1.3.1
Immunoglobulin Structure
(From Johnstone & Thorpe, 1987)

Figure 1.3.1 shows the generalized structure of an IgG molecule. The position of disulphide bonds is indicated by S - S. The sites of papain (yielding two Fab fragments and one Fc fragment) and pepsin (yielding an $F(ab')_2$ fragment and a pFc' fragment) cleavage are shown.

an Ag binding site (i.e. two per Ig molecule). The immense structural diversity allowed by the hypervariable regions (coupled with the variation in the shape and nature of the surface of the binding site thus facilitated), is responsible for the almost unlimited Ag binding specificities available to Abs.

The structure of the remaining domains (the constant (C) domains) is much less variable. Variation in the C_H and C_L domains results in the differentiation of Ig molecules into H chain classes (and subclasses) and L chain types respectively. The F_c region (i.e. the C_{H2} and C_{H3} domains) is responsible for the interaction with various cells and effector systems of the immune system (Burton, 1985), with differences existing between the classes in their interaction with these systems.

1.3.2 The Genetic Basis of Antibody Diversity.

Immunoglobulin L and H chains are encoded by three unlinked gene families (i.e. one for each K and L chains and one for H chains) each containing variable (V) and constant (C) genes. The I_K , L and H chain genes are located on mouse chromosomes 6, 16 and 12 (2, 22 and 14 in humans) respectively (Honjo, 1983).

At the DNA level of a mature B cell, each V and C domain is coded for by a separate, single exon. This is not, however, the case in the germline (Taussig, 1988). Each completed V gene is assembled from individual germline segments, for the L chain these are the V_L and J_L (joining) segments (Bernard et al. 1978; Max et al. 1979 and Sakano, 1979) and for the H chain the V_H , D (diversity) and J_H segments (Early et al. 1980 and Sakano et al. 1981).

The K germline locus of the mouse consists of a single C_K gene, at least 200 V_K segments (Cory et al. 1981) and five J_K segments (one of which is a non expressed pseudogene (Honjo, 1983)). The locus in mice (where is expressed

in only 5% of Ig molecules) has only two V segments, each followed downstream by two J - C segments (Honjo, 1983).

The H chain locus of the mouse consists of the C_H genes (in the order 5' - C_γ - C_δ - C_{γ3} - C_{γ1} - C_{γ2b} - C_{γ2a} - C_ε - C_α- 3') (Gough & Cory, 1986), four J_H segments (Shimizu et al. 1982), 10-20 D segments (Taussig, 1988), and the V_H families (of which nine have been identified and which comprise 1000-2000 genes (Taussig, 1988)).

The rearrangement of the germline genes begins at the stage of pre-B cell development in foetal liver and adult bone marrow (Wall & Kuehl, 1983). The sequence of events is as follows (Alt et al. 1986):

i). the V_H gene is assembled, a productive V_HDJ_H rearrangement leads to expression of cytoplasmic μH chains which terminates further V_H gene assembly (allelic exclusion) and activates V_K to J_K rearrangement;

ii). L chain alleles are sequentially rearranged, K before ;

iii). a protective V_K - J_K assembly leads to expression of K light chain and complete (monomeric) IgM, which terminates further light chain rearrangements (allelic exclusion).

The sequence of events from germline DNA to the expression of L and H chains is depicted in Figure 1.3.2.

Several mechanisms contribute to the diversity of the Ab repertoire (Taussig, 1988):

i). rearrangements of the many V_H, D, and J_H or V_L and J_L segments;

ii). variation in the recombination site, thus generating different codons at the junction between each of the segments;

iii). the enzymatic addition of bases (N sequences), not found in the germline, at the D and DJ junctions (Alt & Baltimore, 1982);

iv). the combinations of V_H and V_L and

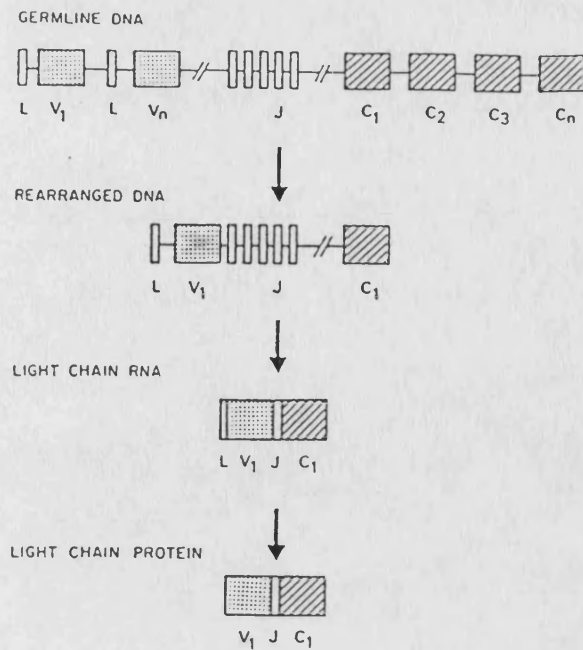
v). somatic mutation as the immune response matures (French et al. 1989).

Figure 1.3.2
Immunoglobulin Genes and Their Expression
(From Reeves, 1987)

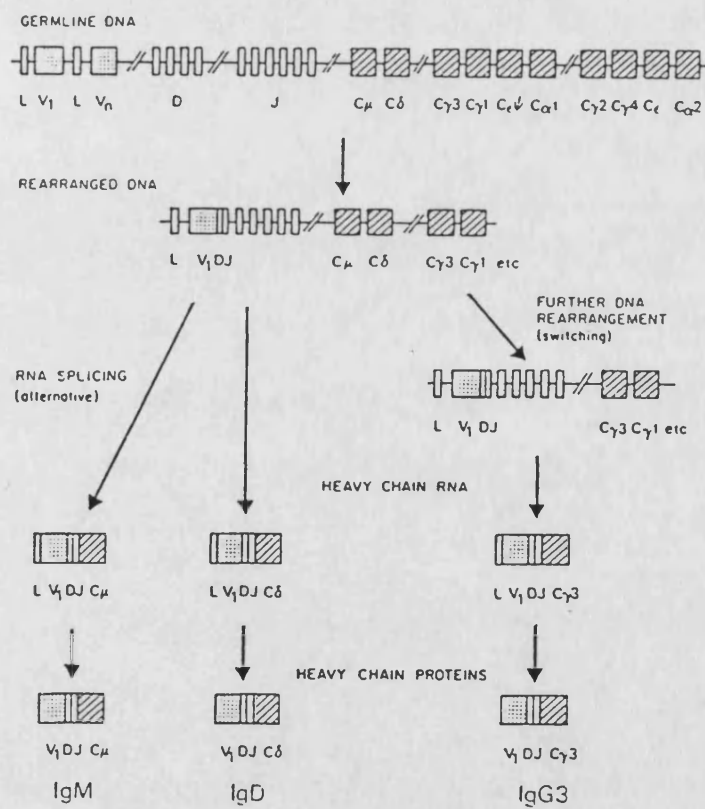
The arrangement of the germline genes (and the events leading to their expression) for human immunoglobulin light (Kappa) and heavy chains are shown in Figure 1.3.2 (a. and b. respectively).

KEY:

C : constant region;
V : variable region;
D : D segment;
J : J segment;
L : Leader sequence.



a). Immunoglobulin light chain (Kappa) genes.



b). Immunoglobulin heavy chain genes.

Even discounting sequences and somatic mutation, the combinational possibilities allow for the generation of about 10^7 different Abs (Taussig, 1988).

Extensive somatic mutation results in the production of higher affinity Abs. This process is triggered when B cells expressing low-affinity surface Ab are stimulated by Ag to interact with T cells and Ag-presenting cells and first becomes effective in the late primary response (French et al. 1989).

After interaction with Ag, the B cell can follow a number of different pathways. It can terminally differentiate into an IgM-secreting plasma cell; it can switch its fully assembled V region to a different C-region gene to express a new class of Ab (Shimizu & Honjo, 1984) or it can become a memory cell.

1.3.3 The Hybridoma Technique.

Polyclonal antisera (pAbs) produced by conventional means (i.e. injection of immunogen followed by collection of blood) will contain a wide variety of Ab structures directed against the immunogen, each different Ab structure being the product of a distinct clone of B cells. Because the components of such antisera will differ with time and individual animals, it is impossible to produce antisera of the purity and reproducibility required of a true chemical reagent (Milstein, 1986). While it is possible to render pAbs antigen specific (e.g. by affinity chromatography (Hudson & Hay, 1980; Johnstone & Thorpe, 1987)), the different molecular species present cannot be separated from each other.

The "purification" achieved by the hybridoma technique is at the cellular rather than the biochemical level and is based upon the production and selection of immortal Ab producing hybrid cell lines (hybridomas), derived from the fusion of individual Ab producing lymphocytes with cells

from an immortal myeloma cell line.

There have been many modifications to the original technique of Kohler & Milstein (1975) (e.g. Kennett et al, (1978); Fazekas de St.Groth & Scheidegger (1980); Galfre & Milstein, 1981; Brown & Ling (1988)), but most follow the same basic approach.

The basic procedure is shown in figure 1.3.3.

The source of Ab producing cells most often used is the spleen. However, the number of B lymphocytes capable of producing specific Ab for any given Ag represents only a minute fraction of the total population in the spleen (Tjissen, 1985). The first step therefore is to enrich the specific fraction by immunization of the animal (usually mouse or rat) with the Ag of interest.

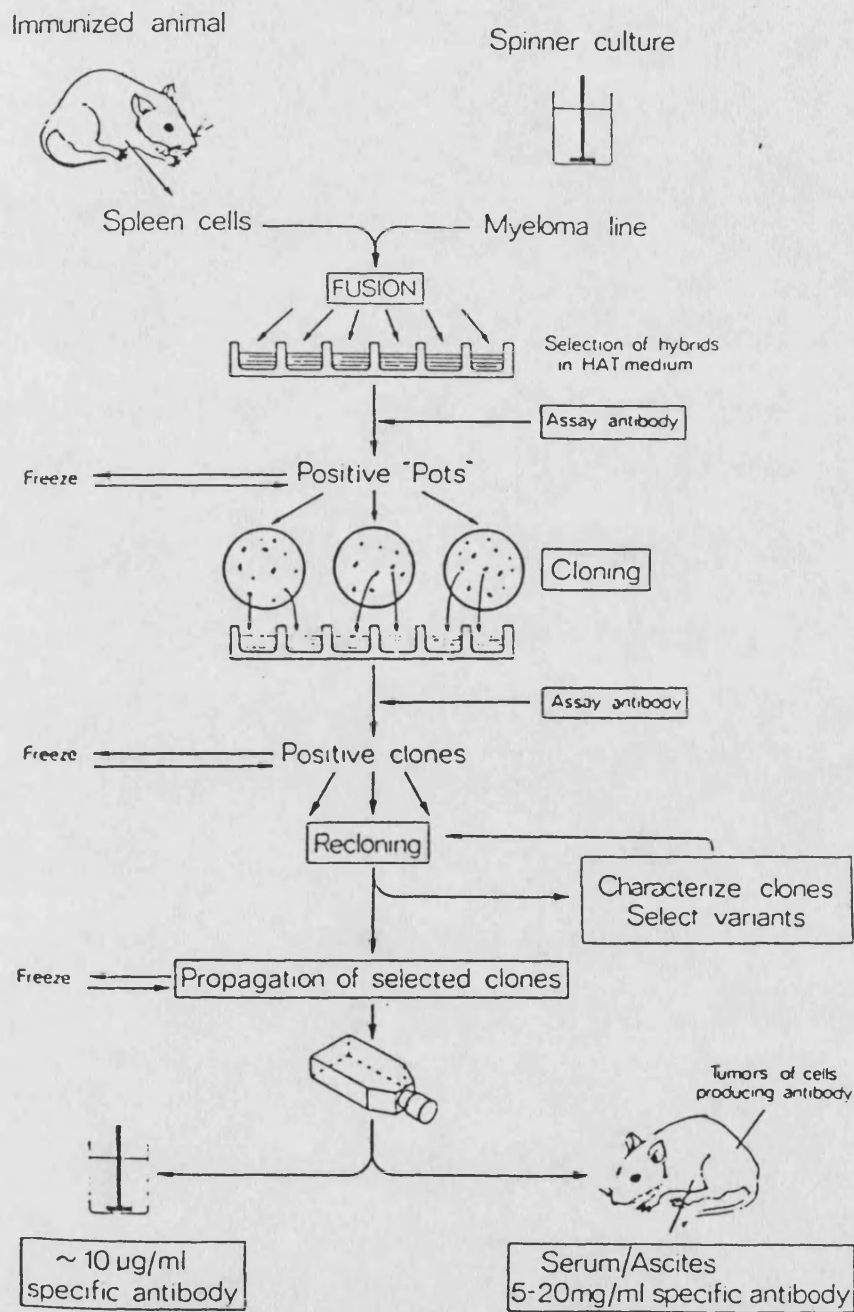
While there are no absolute rules governing immunization protocols, cell fusion should be performed 3 to 4 days after the final Ag boost (Goding, 1980). This is thought to be due to the preferential fusion of actively dividing cells (i.e. Ag stimulated B lymphocytes) (Andersson & Melchers, 1978).

Fusion of isolated spleen cells with a suitable myeloma line (usually derived from the same species) is mediated by polyethylene glycol and is followed by incubation of the cell mixture in a selective medium.

The number of hybrid cells is low in comparison to the parental cells which must therefore be eliminated. The spleen cells do not survive for long in culture, whereas the myeloma cells will proliferate and must therefore be selectively killed.

The selective medium most frequently used contains hypoxanthine, aminopterin and thymidine (HAT medium) (Littlefield, 1964). Aminopterin (which is a folic acid analogue) blocks de novo synthesis of purines and pyrimidines by inhibition of dihydrofolate reductase, and in its presence, cells must use the salvage pathway which utilizes exogenous nucleosides. The pyrimidine pathway involves the enzyme thymidine kinase and requires exogenous thymidine, whereas the purine pathway uses the enzyme hypoxanthine phosphoribosyl transferase (HPRT) and exogenous hypoxanthine.

Figure 1.3.3
Procedure for the Production of Monoclonal Antibodies



(From Galfre & Milstein, 1981)

The use of a myeloma line deficient in one or both of these enzymes (most suitable rodent cell lines are HPRT negative) prevents its growth in HAT medium. Hybridomas are able to survive as they possess the ability to grow in culture (from the myeloma parent) and the HPRT enzyme (from the lymphocyte parent).

An alternative selective medium, Haza (Karsten and Rudolph, 1985), which contains hypoxanthine and azaserine, is sometimes used instead of HAT. Azaserine only inhibits purine biosynthesis thus removing the need to supplement the medium with thymidine. Thymidine depletion by mycoplasma contaminants can be a cause of the failure of hybridomas to grow and therefore the use of Haza, rather than HAT, at the selection stage, allows the survival of hybridomas in the presence of mycoplasma contamination.

The cells produced by fusion are heterokaryons (i.e. they contain multiple nuclei (Ringertz & Savage, (1976) as cited by Milstein (1986)), some of which become synkaryons (i.e. containing a single nucleus with the chromosome content of a heterokaryon) from which emerge some cells that divide and grow. The growth is accompanied by considerable loss of chromosomes and better adaption to culture conditions and eventually results in the establishment of a cell line (Milstein, 1986).

Each cell line, and also subclones taken early after fusion, are likely to have different growth and stability properties and it is therefore important to clone lines as soon as possible after those producing Ab of the required specificity have been identified (Milstein, 1986). This requires the use of an assay that is capable of rapidly and accurately screening large numbers of samples, and can also identify quantitative and qualitative differences between the Ab produced by clones derived from the same line.

Loss of chromosomes can result in the loss of Ab expression and it is thought that such non-producing variants are able to outgrow Ab producing cells of the same clone. This process is more pronounced in the early stages

with more established clones exhibiting more stable characteristics. Frequent screening of expanding clones is necessary and recloning is required if Ab production starts to decrease (Yelton et al. (1978) and Galfre et al. 1980).

Once stable clones producing Ab of the required specificity have been produced, large amounts of mAb can be produced when required (using cells previously frozen in liquid nitrogen) either in vitro by a variety of culture methods (e.g. roller bottles (Griffiths, 1986), spinner cultures (Galfre & Milstein, 1981), hollow fibre culture (Klerx et al. 1988) which gives up to 100 μ g/ml of culture medium used, or in vivo by the production of ascitic tumours in histocompatible mice or rats, yielding up to 10ml of ascitic fluid containing mAb at up to 15 mg/ml (Goding, 1980).

As the initial production costs of a mAb can be several times that of a polyclonal antiserum (in addition to the high initial capital costs involved) and is much more time consuming, it is important to consider whether or not mAbs will be sufficiently superior for the intended application.

The relative merits of polyclonal antisera and mAbs are compared in Table 1.3.1.

Table 1.3.1
The Relative Merits of Polyclonal Antisera
and Monoclonal Antibodies

	<u>Polyclonal Antisera</u>	<u>Monoclonal Ab</u>
Cost	Low	Initially High
Specificity	Variable with animal and bleed	Standard
	Partial cross-reactions with common determinants	Unexpected cross-reactions may occur
	Seldom too specific	May be too specific
Determinants Recognised	Many	Single
Affinity	Variable with bleed	May be selected
Yield of Useful Ab culture	Approx. 1 mg/ml	Up to 100 µg/ml in
		Up to 15 mg/ml in ascitic fluid
Contaminating Immunoglobulin (of the same species)	High	None in culture
		0.5-1 mg/ml in ascitic fluid
Ag purity required	Either pure Ag or serum absorption	Purity desirable but not essential

(adapted from Campbell, 1984 and Tjissen, 1985).

1.3.4. Properties of mAbs

The variability of antisera preparations from different bleeds and animals means that each batch has to be standardized before use whereas mAbs require only initial standardization, all subsequent batches having the same properties (e.g. reactivity profile, storage stability, etc.). In this respect, mAbs possess a distinct advantage especially when the Ab reagent is required in large amounts over a long period. However, caution must be exercised when handling mAbs as they may differ from the average polyclonal Ig of the same subclass in physical and chemical properties such as sensitivity to freezing and thawing and changes in pH (Mosmann et al. 1980).

A polyclonal antiserum will contain a variety of Abs to each of several determinants of an Ag, with the likely consequence of cross-reactivity occurring with Ags possessing one or more of the same or similar determinants (Goding, 1986). Monoclonal antibodies however, can be selected that are specific for a determinant unique to the Ag. This is a feature of mAbs that particularly favours their use in many applications such as diagnosis and therapy (e.g. in targeting specific drugs or toxins) where absolute specificity of binding is required.

While it is possible to remove cross-reacting Ab from a polyclonal antiserum by absorption against the cross-reacting Ag, the resulting preparation would still be a mixture of Abs with the possibility of cross-reactivity against other, untested Ags. In addition, cross-absorption does not allow the isolation of Abs of a single specificity.

Several instances have been described (Lane & Koprowski, 1982) where mAbs have reacted with two apparently unrelated Ags. It was suggested that this may be due to structural similarities between the two Ags. In such situations, it is possible to have 100% cross-reactivity with a mAb whereas an antiserum would also

contain Abs directed to other determinants and would thus exhibit much lower cross-reactivity.

Potential cross-reactivity of this kind can be identified by thorough screening of mAbs against all potentially cross-reactive Ags likely to be encountered within the system. However, this may not always be possible, e.g. when the mAb is intended for therapeutic use in vivo where the number of Ags requiring testing is high.

Similarly, mAbs should, if possible, be selected by the assay in which they are to be applied, as their apparent specificity may differ between assays. Milstein et al. (1983) found that an anti-serotonin mAb bound to dopamine in solution, but not when it had been fixed to tissue with formaldehyde. This was because the amino group of dopamine involved in fixation (cross-linking to protein) is also involved in the determinant recognised by the mAb. Serotonin however, can be cross-linked to protein via other reactive sites, and thus the mAb specifically recognises serotonin in immunohistochemistry.

If mAbs are required to be used in other assays, it is necessary to assess their specificity in the new assay format.

On the other hand, mAbs may also be produced that are too specific for their intended application (Goding, 1986). For example, mAbs intended for use in pathogen diagnosis may react with a determinant only found on some strains of the pathogen and would produce false negative results. A polyclonal antiserum however, would contain Abs to specific and common determinants. Thorough screening of mAbs to select for those that react with all strains, and/or the use of mAb mixtures of known specificity should eliminate most potential problems of this kind.

Whereas the specific fraction of an antiserum is contaminated by other host Igs and proteins, mAbs can be produced that contain little or no host protein especially if produced in serum-free medium in culture. Preparation of pure specific Ab for applications such as enzyme

labelling, is therefore much simpler.

One of the greatest advantages of the hybridoma technique is the potential for the production of specific Ab reagents using an impure Ag.

Production of a specific polyclonal antiserum requires either the use of pure Ag or cross-absorption of the resultant serum. Both can be time consuming and would require repeating for each subsequent immunization and bleed.

In theory, mAbs could be produced to a minor component of a mixture by selection using a suitable differential screening assay. However, because of the likely immunodominance of some Ags in the mixture, some degree of purification of Ag is likely to be required. This could be achieved by a sequential series of fusion experiments, using mAbs produced to immunodominant Ags to remove those Ags from the immunizing mixture for the next fusion (Milstein, 1986).

1.3.5. Applications Of mAbs

MAbs have been used in many areas of science (including clinical and veterinary science, microbiology, and plant pathology) for a variety of purposes such as identification and diagnosis of pathogens and disease, classification of micro-organisms, studies of structure and function, affinity purification, and prophylaxis and therapy. These applications are detailed in many papers and texts including those of Hubbard & Marks (1988), McMichael & Fabre (1982), Tiffin (1987), Halk & de Boer (1985), and Macario & Conway de Macario (1985a & 1985b).

1.3.6. Future prospects for mAbs

The advantages of permanent supply and chemical reproducibility offered by mAbs are likely to result in them replacing pAbs in many instances, particularly in the case of commercial reagents (Milstein, 1986).

The lower affinity of individual mAbs as compared to pAbs has often been cited as a disadvantage in their application in sensitive assays (Milstein, 1986). However, the higher affinity Abs (which may comprise only a small fraction of the total) tend to dominate the specificity of a pAb (Goding, 1986), and it has been demonstrated that mAbs of comparable affinity/avidity can be produced by persistence and the correct methodology (Lowe et al, 1984).

In addition, mixtures of mAbs can be used in which synergistic effects may result in an increase in avidity (Thompson & Jackson, 1984). This approach may not always be successful and has given conflicting results (Ehrlich et al, 1982; Yolken, 1982).

The basic hybridoma technique has allowed the production of a variety of novel Ab reagents (Williams, 1988), that could not be produced by conventional means.

Bispecific Abs are Ig molecules able to react with and link two distinct Ags, and have potential in both diagnosis and therapy either by linking Ag to a label, or to immune effector cells or drugs.

Bispecific Abs can be produced by fusion of two hybridomas producing mAbs of the required specificities (Milstein & Cuello, 1984) and have several advantages over chemically produced conjugates including greater stability and a higher signal-to-noise ratio in immunoassay.

In vivo use of mouse mAbs in humans for diagnosis or therapy may have limited application due to the production of Abs to the mAb by the recipient, which may interfere with binding or increase clearance of the mAb (Houghton, 1988). Hypersensitivity reactions may also occur. Practical difficulties in the production of human mAbs has generally precluded their use (Walls et al, 1988).

The production of "humanized" (chimaeric) Abs by genetic manipulation, either by the insertion of mouse mAb

V region (Morrison et al, 1984) or hypervariable region genes (Jones et al, 1986; Reichmann et al, 1988) of the required specificity into an appropriate human Ig gene may be the solution but there is still the possibility of a host response to the inserted mouse sequences, particularly on repeated application.

Recombinant DNA technology also offers the possibility of producing recombinant Ab-enzyme, or Ab-toxin (immunotoxin) conjugates. The replacement of the F_c with an active enzyme has been demonstrated (Neuberger et al; (1984); Neuberger (1985)), and Chaudhary et al; (1989) produced an active immunotoxin (in E. coli) consisting of a modified Pseudomonas exotoxin and an Ab F_v fragment (i.e. one V_L and one V_H domain). Conjugates produced by this method avoid the potentially denaturing conditions of chemical conjugation and the inherent problems of batch reproducibility.

A recent paper by Hiatt et al (1989) described the production of functional Ab molecules in the leaves of tobacco plants following transformation of tobacco leaf segments with cDNA derived from a mouse hybridoma mRNA. The authors suggested that accumulated Ab molecules (which do not pass through cell walls) could be used to bind and retain environmental contaminants within such transformed plants.

Another recent advance is the production of "single domain Abs (dAbs)" (Ward et al; 1989). DAbs are isolated V_H domains and have been shown in many cases to retain similar Ag binding affinity to the parental molecule. The dAbs were produced in E. coli using libraries of V_H genes cloned from spleen genomic DNA of immunized mice. DAbs can be produced without the need for tissue culture and within a much shorter time period. The affinity of dAbs can be improved by site -directed mutagenesis (Ward et al; 1989). The authors suggested that co-expression of the V_H domains with a repertoire of V_L domains (followed by screening for domain association and Ag binding) could be used to construct F_v fragments or complete Abs. This methodology may be the means by which the effective production of human mAbs is achieved.

1.4. IMMUNOLOGICAL ASSAYS

A wide range of assays are available for the application of Abs in diagnosis. Most assays fall into one of three basic categories, namely those based on precipitation, agglutination and binding assays utilizing labelled Ab or Ag.

The choice of assay to be used is dependent upon many factors including the nature of the Ag, the material to be tested (i.e. host material), whether quantitative or qualitative data is required, where the assay is to be performed, and quality of the Ab available.

1.4.1. Precipitation Based Assays

The reaction between multivalent macromolecular Ags and specific Abs in solution can result, when the reactants are present in the correct proportions, in the precipitation of Ag-Ab complexes. Conditions of Ag or Ab excess will generally not result in precipitation (Ouchterlony & Nilsson, 1978).

Precipitation reactions can be made quantitative and have been used for the measurement of Ab and other serum proteins. A modification that has also been used quantitatively is the measurement of small aggregates formed by mixing dilute solutions of Ab and Ag by nephelometry (Deverill & Reeves, 1980).

Precipitation reactions can also be performed in agarose gels (Ouchterlony & Nilsson, 1978). Ouchterlony double diffusion (ODD) allows the visualisation of precipitin bands formed at the point where diffusing Ab and Ag (placed in separate wells cut in the gel) meet in optimal proportions. ODD can also supply information as to the relatedness of two Ags. Single radial

immunodiffusion involves the diffusion of Ag out of a well into gel containing a suitable Ab dilution. A precipitate ring is formed at the point where equivalence is reached. By including suitable Ag standards this method can supply quantitative data and is routinely used in clinical immunology, particularly for Ig determination (Catty & Raykundalia, 1988).

Precipitation assays are not widely used for diagnosis of plant pathogens although Schaad (1979) cites several examples of the use of ODD for the differentiation and identification of plant pathogenic bacteria, and De Boer (1983) used it for confirming bacterial ring rot diagnosis in potatoes.

While simple to perform, precipitation assays suffer from several disadvantages for the diagnosis of plant pathogenic bacteria; they are not particularly suited to the rapid screening of multiple samples (precipitates take 24-48 hours to develop); sensitivity is limited due to the requirement for sufficient Ag to allow formation of a visible precipitate; they require Ag to be in a soluble form, which, unless a soluble target Ag is present, means that preparation of a suitable sample is required; and, because precipitation assays require the cross-linking of multiple epitopes on the Ag they are not well suited for use with mAbs (especially single ones, i.e. at the screening stage) (Goding, 1986).

1.4.2. Agglutination Based Assays

Agglutination results from the cross-linking of surface Ags on cells or large particles. IgM Abs are generally more efficient at agglutination than IgG because of their multivalency (Goding, 1986).

Slide agglutination, involving the mixing of Ab solution and bacterial suspension on a slide, is a rapid identification method giving results in a matter of

minutes. However, it is inherently limited in its sensitivity due to the requirement for enough bacteria to form visible clumps. A more sensitive modification of this method is to use specific Ab bound to protein A on the surface of Staphylococcus aureus (Kronvall, 1973).

Passive agglutination of specific Ab coated particles (usually red blood cells or latex beads) by Ag allows rapid testing, and has the potential to approach the sensitivity of enzyme labelled Ab techniques (Coombs et al, 1987).

Latex agglutination has been applied to plant pathogens (Slack et al., 1979; Fletcher and Slack, 1986), but the detection limits obtained (10^6 to 10^7 organisms) are not as low as those obtainable with assays such as ELISA.

1.4.3. Binding Assays Utilizing Labelled Ab or Ag

This general heading includes a wide range of assays with binding of Ab and Ag either occurring in the liquid phase or with one or the other component bound to a solid phase. This type of assay can also include binding to Ag in sections of tissue (immunohistochemistry). The most frequently used labels are radioisotopes (radioimmunoassay (RIA)), enzymes (enzyme-linked immunoassay (EIA)) and fluorochromes (immunofluorescence assay (IFA) and fluorometric immunoassay).

The use of enzyme labels is preferable to radiolabels for several reasons including the potential hazards associated with radiolabels, the high cost and short shelf life of radiolabelled reagents (especially compared to EIA reagents) and the level of equipment required to perform RIA (Yolken, 1982). In addition, enzyme immunoassays offer comparable sensitivity and detectability to RIA, can be performed very quickly, and are feasible for use under field conditions.

The fluorescein-linked immunosorbent assay described

by Cole et al. (1982) had greater sensitivity than ELISA, but it does not allow reading of results by eye, requiring a fluorometer for quantification of the fluorescence and has not replaced EIA.

Detection of Ags, particularly bacteria, in tissue by IFA allows the visualisation of individually labelled cells against a dark background.

Only the assay types used in this project will be explained in further detail.

1.4.3a. Enzyme Linked Immunoassays

Two main types of solid phase enzyme linked immunoassays were used in the project, enzyme-linked immunosorbent assay (ELISA) and dot-immunobinding assay (DIA), the main difference between the two being the nature of the solid phase (polystyrene microtitre plates for ELISA, nitrocellulose or nylon membranes for DIA).

A third variation, the slide immunosorbent assay (Conway de Macario et al. 1986), which uses multiwell teflon coated glass slides as the solid phase was tested for rapid hybridoma screening. Despite giving results in approximately one hour, it proved to be unwieldy for the testing of large samples (i.e. more than 100) and was not used further (results not presented).

Many enzymes have been used with the most frequently used being horseradish peroxidase (HRP) and alkaline phosphatase (ALP). HRP is most frequently used in animal applications (where endogenous ALP may be present) and ALP in plant applications (where endogenous peroxidase may be present). However HRP was used throughout this project with no problems. HRP conjugated Abs are cheaper than ALP conjugates and substrate conversion is much quicker. Both enzymes have a number of soluble and insoluble chromogenic substrates, many of which are mutagenic or carcinogenic (Voogd et al, 1980).

The most commonly used ALP soluble substrate is the colourless p-nitrophenyl phosphate which is hydrolysed to yellow p-nitrophenol. The basic substrate for HRP is hydrogen peroxide, the cleavage of which is coupled to the oxidation of a hydrogen donor (the chromogen).

The HRP substrates used in this project (tetramethylbenzidine (TMB) (Bos et al, 1981) for ELISA and 4-chloro-1-naphthol (4CN) for DIA) are both non mutagenic.

ELISA: Immunoassays employing enzyme labels were first described in 1971 by Engvall & Perlmann and van Weeman & Schuurs. The term ELISA includes a wide variety of assays which are all based upon the measurement of the binding of enzyme linked Ab or Ag to immobilised Ab or Ag (all examples given in this section will be where Ab is enzyme linked).

The two basic variants are non-competitive assays and competitive assays which can be further subdivided, depending upon whether the Ag is immobilised directly to the surface of the solid support (direct binding ELISA) or via a specific Ab "trap" coated to the surface (double Ab sandwich (DAS-ELISA)) (Clark et al, 1986) see figure 1.4.1. Assays where the binding of the Ag to the solid phase is stabilised by a spacer molecule such as poly-L-lysine (PLL) are considered to belong to the direct binding type of assay.

Additional subdivision is based upon whether the bound Ag is detected directly with enzyme labelled specific Ab (direct ELISA) or by an anti-species enzyme labelled second Ab binding to Ag bound specific Ab (indirect ELISA).

A variety of general detection systems other than labelled 2nd Ab are also available including enzyme labelled protein A and biotin-avidin systems (generally using biotinylated 2nd Ab and enzyme labelled avidin) (Tijssen, 1985).

Figure 1.4.1

ELISA Variations

(Adapted from Kemeny & Chantler, 1988)

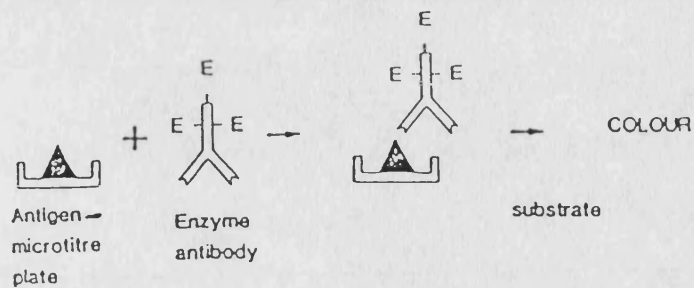
The basic variations in ELISA protocols are represented diagrammatically in Figure 1.4.1.

1. Direct DAB-ELISA: Ag, adsorbed to the surface of a microtitre plate well (i.e. direct Ag binding (DAB)) is detected by the binding of Ag-specific enzyme-conjugated Ab (conjugated enzyme is represented by E);

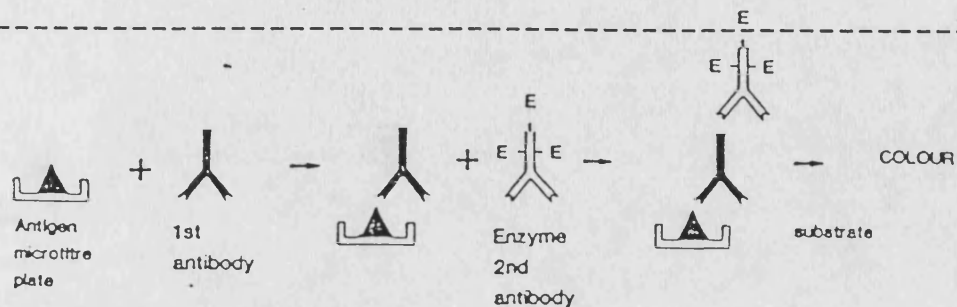
2. Indirect DAB-ELISA: the binding of specific Ab (1st Ab) to Ag is detected by an enzyme-conjugated Ab (2nd Ab) with specificity for the 1st Ab.

3. Direct DAS-ELISA: Ag is bound by Ag-specific Ab (1st Ab) adsorbed to the microtitre plate, and detected by the binding of Ag-specific enzyme-conjugated Ab (2nd Ab). In the example shown the 1st and 2nd Abs are specific for different epitopes on the Ag which is univalent for each epitope. The same Ab could be used in both steps if the Ag was multivalent. Indirect DAS-ELISA can also be performed (see text for details).

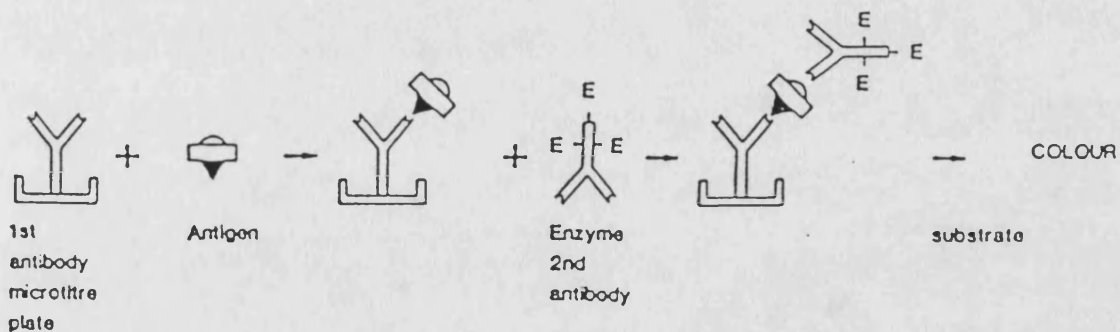
4. Competitive ELISA: the example shown uses enzyme-conjugated Ab and adsorbed Ag to assay free Ag of unknown concentration. When no free Ag is present, Ab binds to adsorbed Ag (as in 4b.). Addition of free Ag results in competition between free and bound Ag for Ab binding sites (as in 4b.). Quantitative results (measuring free Ag) can be obtained by constructing a standard curve from results where known amounts of free Ag are added.



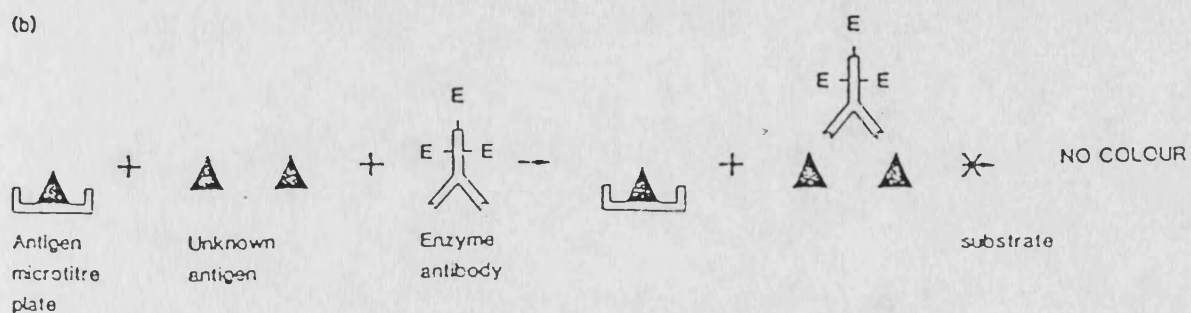
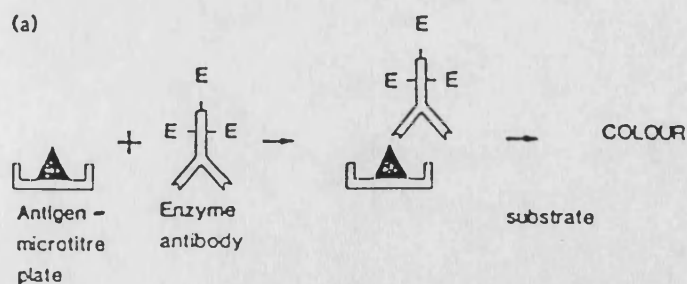
1. Direct DAB-ELISA.



2. Indirect DAB-ELISA.



3. Direct DAS-ELISA.



4. Competitive ELISA.

The choice of assay format to be used is dependent upon several factors including the nature of the Ag being assayed, the avidity/affinity of the antiserum/Abs available and the sensitivity, reproducibility and simplicity required of the assay. It is probably best to try the simplest protocol first and modify it in successive stages if necessary.

Competitive assays are used much less frequently for diagnostic applications than are non-competitive assays. There are several reasons for this, including:

i). the poorer accuracy and sensitivity of competitive assays (Tijssen, 1985; Yolken & Stopa, 1980);

ii). competitive assays measure the decrease in labelled Ab binding to solid phase Ag and thus require comparison of values obtained with and without competing Ag, thereby increasing the complexity of the assay;

iii). results cannot be read by eye (unless complete inhibition occurs) as they rely on a decrease rather than an increase (as in non-competitive assays) in colour;

iv). direct competitive assays (i.e. not using a labelled 2nd Ab) require the incubation of enzyme labelled Ab in biological fluids which may contain inhibitors, proteases etc. which could affect the activity of the enzyme (Clark and Engvall, 1980; cited by Clark et al., 1986).

Binding to the solid phase via a specific Ab trap (i.e. DAS-ELISA) is necessary for some viruses which break up on direct binding (Halk and De Boer, 1985). It may also be of use with other Ags where non-specific material is present in the sample which may inhibit binding or compete for binding space. Indirect DAS-ELISA requires Ab preparations from two different species (unless an $F(ab')_2$ preparation of detecting Ab is used for trapping (Barbara & Clark, 1982)), thus increasing the complexity of the assay. In addition, the DAS-ELISA negates the advantage of unlimited reagent supply allowed by mAbs unless mAbs can also be used to trap Ag, in which case two or more specific

mAbs are required (unless the Ag is multivalent with respect to the relevant epitope). Direct binding assays may therefore be preferable due to their greater simplicity.

Direct ELISAs utilising labelled specific Ab are quicker and generally result in lower non-specific backgrounds (due mainly to reduction in the number of steps), but indirect assays are generally favoured as they remove the need for enzyme conjugation to individual Ab preparations (particularly important with mAbs as individual mAbs may lose activity upon conjugation) and provide an amplification step due to multiple 2nd Ab molecules binding to the Ag bound specific Ab (Yolken & Stopa, 1980; Barbara & Clark, 1982).

ELISA can be used to give both qualitative and quantitative results.

Qualitative assays, based on an "all or nothing" response with test samples, require the establishment of a positive/negative threshold value based on results obtained with known negative samples. It is important to use a number of different negative samples to ensure adequate coverage of the possible range of healthy values. In addition, the negative samples should be of the same type as the test samples (e.g. for testing plant material, the negative and test samples should be matched with respect to such factors as cultivar, rootstock, age, growing conditions etc.).

Sutula et al. (1986) surveyed several major plant science journals for ELISA related articles and found great variation in the methods used to determine threshold values. They suggested the construction of a histogram to display the frequency distribution of ELISA absorbance values obtained with characterized positive and negative samples. The ELISA system that they tested produced a bimodal distribution with the two populations being well separated, suggesting that the assay could be confidently used. Data interpretation problems would be expected however, if there was less of a distinction between the two

populations.

The construction of a histogram does not solve the problem of setting the threshold, however, and they examined the effect on the production of false positive and negative results of several methods for setting the threshold. The results suggested that the method chosen should reflect the relative importance of false negative or positive results in the assay as methods producing higher threshold values increase the percentage of false negative results and decrease the percentage of false positives, and vice versa.

For an assay detecting an infectious disease agent within a crop with a view to eradication of all infectious material, it is probably best to select a low threshold and accept that some uninfected plants will be destroyed. The use of twice the mean value of the negative controls produced no false negative results in the example cited. This method of setting the threshold was adopted for use in this project.

A quantitative ELISA should include a range of known Ag concentrations to allow construction of a standard curve. The treatment of the standard samples should be identical in all respects to the test samples. While unlikely to be a problem with polyclonal antisera, quantitative determinations using mAbs to organisms such as bacteria may not be reliable due to the likelihood of quantitative and qualitative variations in surface Ags between isolates. Mixtures of several mAbs reactive with a range of surface Ags may, to some extent, counter the problem.

Dot Immunobinding Assay: DIA is based on the general ELISA principle and was first described by Hawkes et al in 1982. Ag is usually immobilized directly onto the solid phase (usually nitrocellulose or nylon membrane), although DAS procedures are possible (Powell, 1987). Detection of bound Ag is achieved by using a chromogen with an insoluble reaction product, resulting in the deposition of colour

onto the membrane. DIA is best suited as a qualitative test, but can be made quantitative by using reflectance densitometry, alternatively estimations can be made by eye by comparing colour intensity of test samples with suitable standards.

A number of variations of the DIA procedure have been described (Towbin and Gordon, 1984). For hybridoma screening, the membrane can be ruled into a grid and 1 μ l aliquots of Ag applied to each square. 1 μ l aliquots of hybridoma supernatant can be applied to each dot (after drying and blocking the membrane), and, after a short incubation (3 to 5 minutes) bound Ab can be detected by immersion of the membrane in 2nd Ab solution followed by substrate (Campbell, 1984).

For larger volumes of sample, an apparatus such as that described by Domin et al (1984) and Smith et al. (1984) (e.g. the Bio-Dot apparatus (Bio-Rad) can be used, which allows incubation of up to 300 μ l Ag in a 96 well format in which the base of the wells is formed by the membrane. This is particularly useful for dilute samples as it allows concentration of the Ag in a defined dot by connecting the apparatus to a vacuum pump to draw the solution through the membrane.

DIA is a particularly useful method for application in situations where facilities for ELISA are not available as it requires no expensive equipment. In addition, Ag coated membranes are stable and can be stored for a considerable time before probing, making the method especially suited for field sampling for testing later in the laboratory (Towbin & Gordon, 1984).

1.4.3b. Immunofluorescence Assay (IFA)

Antigens in tissue sections or cell smears can be specifically identified by microscopical examination by using IFA (Johnson et al, 1986; Goding, 1986). The basis of IFA is linkage of a fluorochrome either to specific Ab (direct IFA) or 2nd Ab (indirect IFA) and visualisation of bound Ab by fluorescence of the fluorochrome when subjected

to the appropriate wavelength of light.

The most commonly used fluorochromes are fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC). FITC has an absorption maximum at 495nm (blue) and an emission maximum at 525 nm (apple green) as compared to 555nm (green) and 580 nm (orange-red) respectively for TRITC.

An alternative fluorochrome, R-Phycoerythrin, (absorption range from 475 nm to 575 nm (maximum at 566 nm), with an emission maximum at 574 nm) is potentially more sensitive than FITC and TRITC due to its relatively large extinction coefficient and quantum efficiency (Kronick, 1986). Another factor which can determine the sensitivity of fluorescent measurements is the background signal due to fluorescent molecules in the sample. This is most severe with excitation in the blue end of the visible spectrum, and is therefore a particular problem with FITC (Soini & Hemmila, 1979).

IFA has been used to examine bacteria both in plant material (Brlansky et al. 1982) and in isolation from plant material (Auger and Shalla, 1974).

As individual bacterial cells can be seen, the limit of detection of IFA is theoretically one cell. In practice, unless the sample can be concentrated into a small area, it may be necessary to examine many fields of view before locating labelled cells in dilute samples.

The relatively time consuming microscopical examination required by IFA may be offset by the shorter incubation times required by the immunoreactants (with respect to ELISA). While requiring an expensive epifluorescence microscope, the additional equipment required for IFA is inexpensive.

A major advantage of IFA over the other assay systems available is that it allows the visualization of bacteria in infected tissue.

1.5. THE APPLICATION OF SEROLOGICAL METHODS TO THE DIAGNOSIS AND IDENTIFICATION OF PLANT DISEASES AND PATHOGENS.

The effective control of any disease usually requires the rapid and accurate identification of the causal agent, either on the basis of symptoms or by isolation or detection of the agent. Once the pathogen has been identified the appropriate control methods can be implemented.

Plant abnormalities cannot always be diagnosed solely on symptoms, as similar pathological conditions may be caused by different agents, e.g., both bacteria and fungi can cause soft rots and wilts, and bacterial soft rots may be caused by species of either Pseudomonas or Erwinia.

There is, therefore, often a need for further diagnosis which generally involves identification of the pathogen responsible.

The amount of diagnostic work that can be undertaken may be limited by time and the resources available, and is also related to need, i.e. whether a rapid diagnosis is required by the grower or if a more comprehensive identification is needed for disease records.

The traditional method of identifying bacteria is isolation (on isolation or selective medium) followed by cultural and physiological testing (for further details see Fahy and Persley (1983)). The differential characters used for the identification of plant pathogenic bacterial genera are summarized in Table 1.5.1.

The time and effort required for diagnosis by such methods are dependant upon many factors including the presence of faster growing saprophytes in the sample, and the time taken for colonies of the pathogen to appear. As can be seen from Table 1.5.1., many of the "differential" characters are shared between, or are variable within genera. Successful diagnosis is therefore dependant on the recognition of multiple correlated features.

Table 1.5.1

Differentiation of the Major Genera of Plant Pathogenic
Bacteria

Table 1.5.1. lists the characteristics that are of use in the provisional determination of the identity of the major plant pathogenic bacterial genera.

KEY:

- + : positive for character
- : negative for character
- v : variation within the genus for the character
(i.e. some species possess it and some do not)
- Ox : oxidative metabolism
- F : fermentative metabolism
- PHB : poly- β -hydroxybutyrate
- ND : not determined

(Adapted from Fahey & Persley, 1983)

<u>Character</u>	<u>Corynebacterium</u>	<u>Erwinia</u>	<u>Pseudomonas</u>	<u>Xanthomonas</u>	<u>Agrobacterium</u>	<u>Xylella</u>
Disease symptoms	Gumming of inflorescences Wilts and/or leaf spots	Vascular wilts Dry necroses Leaf spots Soft rots	Leaf spots Vascular wilts Soft rots	Leaf spots Vascular Wilts Stem cankers	Crown gall Hairy root formation Soil saprophytes	Leaf scorches Vascular wilts
Gram reaction	+	-	-	-	-	-
Motility	v	+	+	+	+	-
Flagella	Few polar or lateral	Peritrichous	One or several polar	One polar	Sparse lateral	Aflagellate
Colony colour	Orange, blue, or yellow	White or yellow	White or yellow	Yellow (rarely white)	White (rarely yellow)	White
Diffusible pigments	Usually absent	Usually absent	Fluorescent or phenazine or absent	Usually absent	Usually absent	Absent
PHB inclusions	-	-	v	-	v	-
Kovacs' oxidase	-	-	v	- or weak +	+	-
Glucose metabolism	Weak Ox or inert	F	Ox	Ox	Ox	Ox
Starch hydrolysis	-	-	v	v	-	-
Nitrite from nitrate	-	v	v	-	v	ND
3-keto lactose	-	-	-	-	v	-
% guanine + cytosine in the DNA	65-75	50-58	58-70	63-69	59-63	51-53

Some pathogens, such as MLOs (and also viruses), cannot be cultured and can only be identified by techniques such as transfer to indicator hosts or electron microscopy.

Identification by such methods is both time consuming and requires suitably trained and experienced staff.

A well designed serological test however, while requiring experienced staff for its development, has the potential for field use by farmers, growers, etc, or to enable the rapid testing of many samples in diagnostic laboratories, and can be applied directly to the infected material or extracts of it.

Serological tests also have the potential to supply further information, e.g identification of strains, pathovars, etc of a pathogen or number of organisms present.

There are also situations such as latent or symptomless disease, stock and seed-borne pathogens (both important in import/export of seedlings and seeds) and vector-borne diseases where examination by conventional means is either impossible or impractical due to limitations of time or quantity or the low numbers of organisms involved. Again, serological assays allowing rapid testing of many samples have great potential in such situations, although many of the assays reported to date (to be discussed later in this section), especially those based on the use of polyclonal antisera, have encountered problems due to cross-reactions or inability to detect low numbers of organisms and require confirmation by other means.

The choice of which assay to use depends on several factors including the level of equipment available (e.g. ELISA and IFA may need expensive equipment), number of samples to be tested, sample preparation required, whether or not quantitative data is required, and the nature and concentration of the pathogen (e.g. IFA would be inappropriate for the detection of low numbers of virus, whereas it may be the most suitable method for detecting low numbers of bacteria).

Whereas polyclonal antisera can generally be applied to any assay, it may be necessary to devise a suitable

assay prior to screening when using mAbs since transfer of mAbs to a different assay may not be successful. Caution is required however, when applying any Ab preparation to a new assay, e.g. an antiserum that appears specific in a double diffusion technique may be cross-reactive when applied to ELISA due to the greater resolution and detectability of the latter.

The use of serology in plant pathology is by no means a recent development, with Jensen in 1918 demonstrating the differentiation of a Danish strain of Agrobacterium tumefaciens from an American strain using agglutination tests. The application of polyclonal antisera since then to the study of phytopathogenic bacteria (Schaad, 1979), and viruses (Van Regenmortel, 1982) is well documented. The production of mAbs to bacteria was first reported in 1978 by Polin and Kennett (as cited by Polin, 1984) who produced mAbs against each of the serotypes of group B streptococcus, but it was not until 1984 that De Boer and Wieczorek reported the production of mAbs to a phytopathogenic bacterium.

The number of reports of the production of mAbs to phytopathogenic bacteria, bacteria-like organisms and MLOs has increased steadily since then (De Boer, 1987), a large number of which deal with fastidious and non-culturable organisms.

Much effort has also been focused on the production of mAbs to phytopathogenic viruses, applications to over 30 having been reported up to 1987, which is probably as much a reflection of the potential of mAbs for the detection of viruses over existing methods as it is of the greater number of plant diseases caused by viruses. Much of the progress to date has been reviewed by Halk and De Boer (1985) and Martin (1987).

MAbs have also been successfully applied in a few cases to fungal plant pathology (reviewed by Oullette and Benhamou, 1987).

At present most applications of mAbs in plant pathology have been for diagnostic or taxonomic purposes. The tendency for advances in medical sciences to often find applications in plant sciences suggests the likely use of

mAbs to probe structure function relationships of particular antigens (Macario and Conway de Macario, 1984; Tiffin, 1987) with a view to understanding the nature of pathogenicity and the relationship between pathogen and host, and possibly even the use of mAbs as prophylactic (and non-toxic) agents.

A search of the literature over the past 20 years shows that there have been many attempts to produce pAbs for use in the diagnosis of diseases caused by plant pathogenic bacteria (summarized in Table 1.5.2). Despite producing antisera that reacted with the target organism, for a variety of reasons several of these studies were unsuccessful in producing reliable diagnostic tests.

Many of the failures were due to lack of specificity of the antisera, including cross-reactivity with host material (Fletcher & Slack, 1986), unrelated plant pathogens (Calzolari et al. 1982), or closely related species (Alvarez & Lou, 1985; Eden-Green, 1987c; Malin et al. 1985). Absorption of the antisera with the cross-reacting Ag can be used to increase the specificity (Mayer & Echandi, 1986; Clark et al. 1983), but may also result in loss of titre to the point where antiserum produces insufficient positive/negative discrimination (Eden-Green, 1987c; Alvarez & Lou, 1985).

Several different assays have been used in confirmation with the pAbs for the detection of plant pathogenic bacteria. The use of assays such as latex agglutination has now largely been usurped by labelled Ab methods (e.g. ELISA, IFA) which offer greater sensitivity and in the case of ELISA, the potential for both quantitative data and the ability to handle large numbers of samples.

Agglutination assays should not be dismissed however, particularly for application in situations where a simple assay is required or where maximum sensitivity is not important. Both Fletcher & Slack (1986) and Oellerman & Pillay (1987) reported the successful use of latex agglutination and haemagglutination respectively.

Table 1.5.2.

**Applications of Polyclonal Antisera to Plant Pathogenic
Bacteria**

<u>Bacteria</u>	<u>Assay</u>	<u>Reference</u>
<u>C. sepedonicum</u>	ODD	De Boer (1983)
<u>C. sepedonicum</u>	LA	Slack et al.(1979)
<u>C. sepedonicum</u>	IFA	De Boer & Copeman (1980)
<u>C. sepedonicum</u>	IFA	Slack et al.,(1979)
<u>C. sepedonicum</u>	IFA	Miller (1984)
<u>S. citri</u>	LA	Fletcher & Slack (1986)
<u>C. xyli</u>	LA	Guzman et al., (1989)
<u>X. campestris</u>	IFA	Daniel & Boher (1981)
<u>E. carotovora</u>	IFA	Allan & Kelman (1977)
<u>X. ampelina</u>	ELISA	Lopez et al., (1987)
<u>X. campestris</u>	ELISA	Alvarez & Lou (1985)
<u>X. campestris</u>	RIA	Malin et al., (1985)
<u>X. campestris</u>	ELISA	Malin et al., (1985)
<u>X. campestris</u>	DIA	Malin et al., (1985)
<u>S. ipomea</u>	ELISA	Moyer & Echandi (1986)
PDB	IFA	Auger & Shalla (1974)
PDB	IFA	Brlansky et al., (1982)
<u>C. xyli</u>	FADC	Davis & Harrison (1989)
<u>C. xyli</u>	DIA	Davis & Harrison (1989)
<u>C. xyli</u>	TB-DIA	Davis & Harrison (1989)
<u>C. xyli</u>	HA	Oellerman & Pillay (1987)
<u>C. xyli</u>	IFA	Oellerman & Pillay (1987)
<u>C. xyli</u>	Aggln.	Oellerman & Pillay (1987)
<u>C. xyli</u>	ELISA	Pillay & Oellerman (1988)
<u>S. citri</u>	ELISA	Clark et al., (1983)
Clover MLO	ELISA	Clark et al., (1983)

Table 1.5.2. summarizes the reported applications of pAbs to the diagnosis of plant pathogenic bacteria over the past 20 years.

The bacterial species involved and the assay used are given.

Assay Abbreviations:

Aggln.	Agglutination
FADC	Fluorescent Ab direct count
HA	haemagglutination
LA	latex agglutination
ODD	Ouchterlony double diffusion
RIA	radioimmunoassay
TB-DIA	tissue blot DIA

A potential problem associated with ELISA is that its increased sensitivity may result in the detection of weak cross-reactions (with other bacteria or plant material) that might not be seen in assays such as gel diffusion or latex agglutination, or could be distinguished from specific reactivity in IFA (Alvarez & Lou, 1985; Malin et al. 1985).

Because of the problems encountered with pAbs, attention in recent years has been focused on the potential of mAbs for use as specific diagnostic reagents. A major advantage of mAbs in this context is that they can be selected for the required specificity by screening against the specific organism as well as cross-reacting organisms and plant material.

A further advantage of mAbs, especially for use in a diagnostic test is that they can be produced as a standardized reagent in practically unlimited quantities. PAbs however, are likely to vary with animal and bleed, and are only available in finite quantities per animal. Repeat batches require standardization of the assay and also more Ag, which in the case of MLOs and other fastidious organisms may be difficult to obtain in sufficient quantities.

The successful production and application of mAbs to plant pathogenic bacteria has been reported by several workers (see Table 1.5.3.).

Table 1.5.3.

Applications of MAbs to Plant Pathogenic Bacteria

<u>Bacteria</u>	<u>Assay</u>	<u>Reference</u>
<u>C. sepedonicum</u>	IFA	De Boer & Wieczorek (1984)
<u>C. sepedonicum</u>	ELISA	De Boer et al. (1988)
<u>X. campestris</u>	RIA/ELISA	Alvarez et al. (1985)
<u>E. carotovora</u>	ELISA	De Boer & McNaughton (1987)
<u>E. amylovora</u>	IFA	Lin et al. (1987)
<u>X. fastidiosa</u>	not stated	Wells et al. (1987)
AY-MLO	ELISA/IFA	Lin & Chen (1986)
<u>S. citri</u>	not stated	Martin-Gros et al. (1987)
Citrus Greening	not stated	Garnier et al. (1987)

Table 1.5.3. summarizes the reported application of mAbs to plant pathogenic bacteria. The bacterial species involved and the assay(s) used are given.

The production of specific mAbs has not always led to the development of successful assays. The failure of such mAbs has been due to a variety of reasons.

De Boer & Wieczorek (1984) produced a mAb specific for C. sepedonicum which despite working well in IFA, reacted poorly in ELISA, which, because of the requirement for the testing of large numbers of potato samples, was considered a more appropriate assay. A subsequent mAb (De Boer et al. 1988) which reacted well in ELISA did not react with other plant pathogenic bacteria but did react with one non-pathogen isolated from potato. The authors therefore advised confirmation of ELISA positive samples by IFA using their first mAb, a solution which is obviously not ideal.

De Boer & McNaughton (1987) produced three mAbs that were specific for E. carotovora subsp. atroseptica

serogroups I and XXII which were able to discriminate between healthy and infected stems in ELISA. However, due to the inability of the mAbs to detect low numbers of bacteria discrimination between tubers (which contain lower amounts of bacteria) from healthy and infected plants was not possible.

Only a single, enzyme conjugated mAb was used, suggesting that the use of all three mAbs in an indirect procedure may have increased the sensitivity of the assay.

Lin & Chen (1986) produced a mAb specific for the non-culturable aster yellows MLO, that unlike pAbs, showed no reactivity with healthy plant material or other MLOs, and was able to detect the AY-MLO in the sieve tubes of AY infected lettuce in IFA. Positive reactions were not obtained with all plants suspected of having AY infection, suggesting that the mAb may have been too specific to identify all isolates.

While at present limited in number, the reports summarized in this section demonstrate the potential and advantages of mAbs over pAbs as specific reagents, particularly in cases where previous pAb reagents had lacked specificity.

The examples cited also demonstrate some of the potential pitfalls of mAbs such as reduced sensitivity, assay restriction, unexpected cross-reactions and narrow specificity. Such problems however, may be overcome by the use of mixtures of mAbs and extensive screening to isolate mAbs of the required specificity.

1.6. THE STRUCTURE AND IMMUNOCHEMISTRY OF THE BACTERIAL CELL SURFACE

1.6.1. The Bacterial Cell Envelope

The cell envelope of all prokaryotic micro-organisms, with the exception of the Mollicutes (Mycoplasmas) consists of an inner cytoplasmic membrane which controls the substrate and electron transport processes of the cell (Garland, 1977) and is the site of biosynthesis of extracellular macromolecules (Rogers et al, 1980); and a strong outer cell wall which maintains the shape of the cell and protects the fragile cytoplasmic membrane from rupture by the high osmotic pressure exerted on it by the cell cytoplasm (Rogers et al, 1980).

The Eubacteria can be divided into three major groups based on the structure of the cell envelope; the Mycoplasmas which do not synthesise a cell wall (Dundas, 1977), the membrane serving as the outer bounding layer; the Gram-positive bacteria, which synthesise a monolayered cell wall; and the Gram-negative bacteria which synthesise a cell wall composed of at least two structurally distinct layers (Starr, et al., 1981).

Electron micrographs of cell wall cross-sections show the wall of a Gram-positive bacterium as a uniform layer (20 to 80 nm in width) with no internal features (Rogers et al, 1980), whereas that of a Gram-negative bacterium is composed of at least two readily distinguishable layers, (each considerably thinner than the wall of a Gram-positive bacterium); a dense, uniform inner layer (2-3 nm wide) (Murray et al, 1985), overlain by a thicker (5-10 nm) outer layer (Glauert & Thornley, 1969). The outer layer has a very similar appearance to the cytoplasmic membrane, although it is very different in chemical composition and function. The surface of the outer wall layer is highly irregular, giving a wavy profile in thin sections and a

rippled appearance to cells visualized by negative staining in electron microscopy, probably due to shrinkage of the cell in the dehydration and straining procedures (Rogers et al, 1980).

The simplest method of differentiating Gram-positive and Gram-negative organisms is by differential staining (Gram stain)(Gram, 1884). Heat fixed smears of growing cells are successively stained with the basic dye, crystal violet, and with a dilute iodine solution. Following treatment with an organic solvent (alcohol or acetone) Gram-positive bacteria remain stained a deep blue-black while Gram-negative bacteria are completely decolorized (and can be envisualized by counterstaining with a dye such as carbol fuchsin).

The dye stains the cytoplasm of Gram-positive organisms, the cell wall being required to prevent its extraction by organic solvents, which is indicative of a basic chemical difference between the cell walls of Gram-positive and Gram-negative cells (Salton, 1961).

The major chemical components of Gram-positive and Gram-negative bacteria are summarized and compared in Table 1.6.1.

Table 1.6.1.

Major Chemical Components of Bacterial Cell Walls.

<u>Component</u>	<u>Gram+ve</u>	<u>Gram-ve</u>	
		<u>Inner Layer</u>	<u>Outer Layer</u>
Peptidoglycan	+	+	-
Teichoic Acid	+	-	-
Polysaccharide	+	-	-
Protein	+ or -	-	+
Lipopolysaccharide	-	-	+
Lipoprotein	-	+ or -	+

Gram-positive bacteria will not be further discussed as the project was concerned only with Gram-negative bacteria.

Only features of potential relevance to the work done in this project will be discussed. Additional information on bacterial surface structure and techniques is presented by Hancock & Poxton (1988), Mayer et al., (1985), Nikaido & Vaara (1985), Rogers et al., (1980), and Salton (1987).

1.6.2. The Structure and Constituents of The Gram-Negative Bacterial Cell Wall

Integral Wall Components: the structure of the cell envelope of a Gram-negative bacterium is represented diagrammatically in Figure 1.6.1.

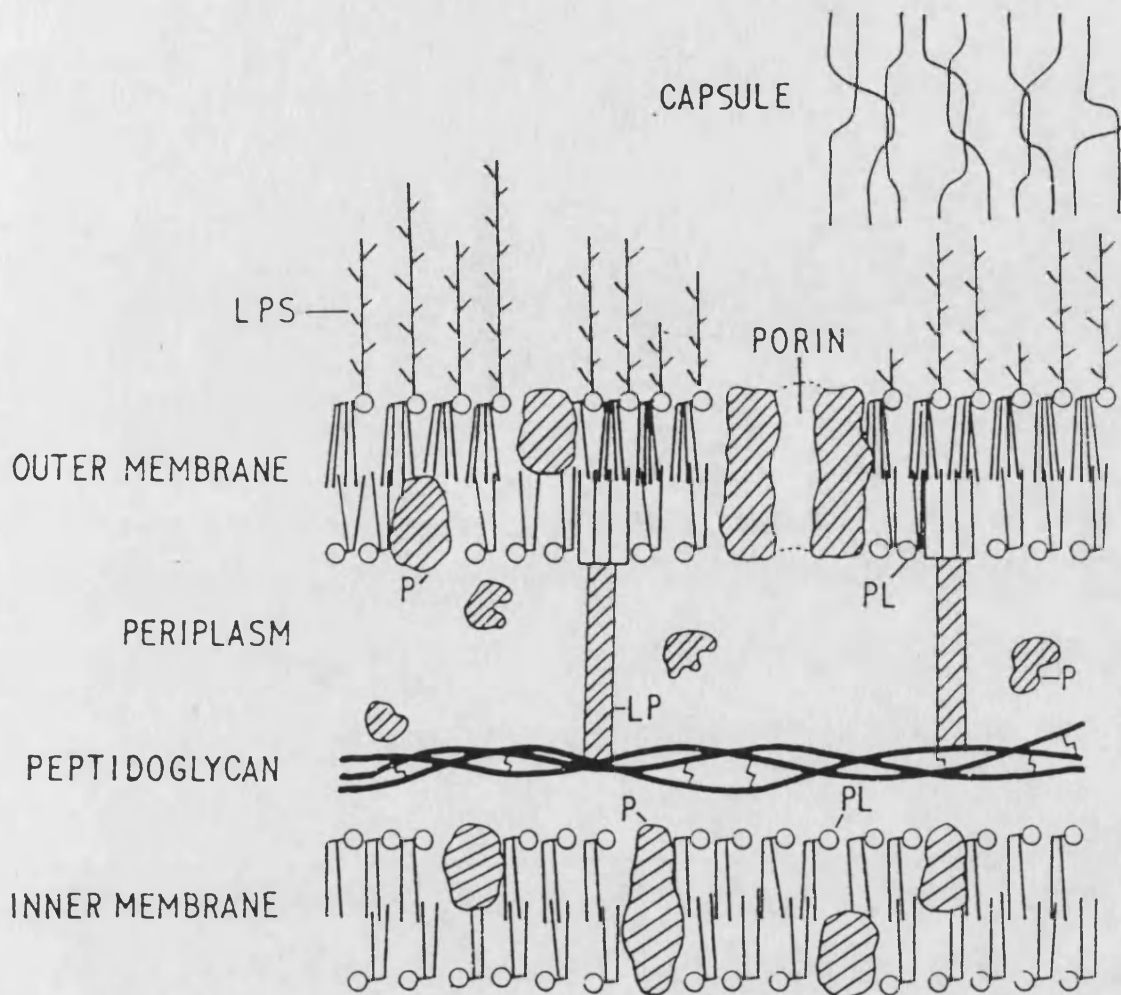
The inner layer of the cell wall corresponds to the peptidoglycan which provides the strength and maintains the shape of the wall. Peptidoglycan is cross-linked through its peptide components to form a single, covalently linked, cell-shaped macromolecule (Rogers et al. 1980). Peptidoglycan consists of three parts: the glycan backbone, the linear muramyl tetrapeptide, and the peptide cross-link, each of which is subject to variation between species or within a species under different growth conditions or differentiation processes (Hancock & Poxton, 1988).

The glycan backbone usually consists of the repeating disaccharide unit (N-acetylglucosamine β 1-4 N-acetylmuramic acid) which is made up of alternating residues of N-acetylglucosamine and its 3-O-D-lactyl ether, N-acetylmuramate, polymerized by β 1-4 glucosidic linkages (Strange & Dark, 1956).

The tetrapeptide is attached to the lactyl group of the muramic acid residues and has a well conserved structure, usually of the sequence L-alanine-D-glutamic acid-AA-D-alanine, where AA is a species specific diamino acid (Schleifer & Kandler, 1972).

Figure 1.6.1

The Cell Envelope of a Gram-Negative Bacterium



KEY:

- LP : Lipoprotein
- LPS : Lipopolysaccharide
- P : Protein
- PL : Phospholipid

(From Hancock & Poxton, 1988)

The peptide cross-link is the most variable feature of the peptidoglycan structure and is a valuable taxonomic character (Schleifer & Stackebrandt, 1983 and Schleifer & Kandler, 1972), although in most Gram-negative bacteria it consists of a direct peptide bond between the side-chain amino-group of the diamino acid of one tetrapeptide and the carboxyl of the terminal D-alanine of a tetrapeptide on another peptidoglycan strand (Kato et al. 1979).

The peptidoglycan is linked to the other part of the cell wall, the outer membrane (OM), by a lipoprotein of low molecular weight (7.2 kDa in *E. coli*) (Inouye et al., 1972). The lipoprotein is covalently linked to the peptide moiety of the peptidoglycan through its C-terminal lysine residue and by an interaction between its N-terminal lipid and the lipophilic region of the OM (Braun & Bosch, 1973).

In *E. coli*, the lipoprotein is present at a ratio of one molecule for every ten disaccharide repeating units of the peptidoglycan.

In intact cells the OM renders (Braun, 1973) the lipoprotein inaccessible to anti-lipoprotein Abs, but after enzymic degradation of peptidoglycan, all the lipoprotein, still attached to fragments of peptidoglycan, is found in the OM fraction (Braun and Bosch, 1973).

When viewed in the EM, the OM in OsO₄ fixed sections has the trilaminar cross-sectional appearance typical of biological membranes, in which the charged groups of the two surfaces become stained while the lipid interior forms a clear band (Glauert & Thornley, 1969, Smit et al. 1975 and Cook et al.). However, whereas the inner (cytoplasmic) membrane (CM) is a phospholipid bilayer (Smit et al. 1975), the OM, although made up of phospholipid on its inner layer, has an outer layer consisting largely of lipopolysaccharide (LPS) (Smit et al. 1975).

In comparison with the CM, the OM contains relatively few different proteins (Bragg & Hou, 1972 and Ames et al. 1974).

Lipopolysaccharides (LPS) are characteristic

components of the cell wall of all Gram-negative bacteria (Westphal et al., 1981), and are localised in the outer layer of the OM (Nikaido & Nakae, 1979). They contribute to the integrity of the OM, and, in the case of enteric bacteria, protect the cell against the action of bile salts and lipophilic antibiotics (Lugtenberg & van Alphen, 1983) and may allow the pathogen to evade the hosts immune system (Nikaido, 1970). The LPS of non-pathogenic bacteria may serve to protect them against phagocytosis by other micro-organisms (Gerisch et al., 1967, as cited by Nikaido, 1970).

The structure of LPS exhibits enormous variability both within and between different genera (Nikaido, 1970). The antigenic properties of LPS ("O Ags") have been exploited in the classification of Salmonella serotypes (Kauffmann, 1966), and other bacteria, including E. carotovora (De Boer et al., 1979 & 1985).

All LPS molecules show the same basic structure, being composed of three distinct regions (see Figure 1.6.2.), the O-specific polysaccharide chains, the core-oligosaccharide, and the lipid A moiety (Luderitz et al., 1966).

LPS has endotoxic properties in higher organisms (Luderitz et al. 1973). Mild acid hydrolysis splits LPS into lipid A (which retains the toxicity and polysaccharide (responsible for antigenic specificity) (Osborn, 1963).

Whereas the polysaccharide portion can be lost by mutation (as in rough strain mutants) without injury to the organism, lipid A deficiency is a conditionally lethal state due to its essential structural role in the cell envelope (Rogers et al., 1980).

The lipid A region is the most conserved part of the LPS molecule (Mayer et al. 1985) and is common (amongst species studied) to all Enterobacteriaceae, Pseudomonadaceae (Rietschal & Luderitz, 1980) and some, but not all, Rhodospirillaceae (Mayer & Weckesser, 1984).

The polysaccharide portion of LPS consists of the core oligosaccharide and the O-antigenic polysaccharide. The core region links the lipid A to the O chains and has the same structure in all Salmonella serotypes (Mayer et al. 1985). In other species (e.g. E. coli) variation in core structure between serotypes has been observed (Mayer et al., 1985).

The O-specific chains are, in most cases, built up of repeating units of lipopolysaccharides which exhibit in strain-specific structural diversity. The sugar constituents, their sequence and their mode of linkage determine the serological O specificity of strains (Luderitz et al., 1966 and Nikaido, 1970).

The O specific chains vary in length even in the same organism (Ryan & Conrad, 1974), and the characteristic series of bands (ladder pattern) seen in SDS-PAGE is thought to reflect this, being due to LPS molecules with different numbers of O-repeating units (Tsai & Frasch, 1982). In many cases a series of doublet bands is observed which may be due to additional heterogeneity in the core or lipid A region (Vaara et al. 1981).

Whereas many taxonomic markers are determined by single or a few genes, LPS is the product of the collaboration of several dozens of enzymes (each of the three regions is under separate genetic control) and therefore has great potential in both intra- and inter-species classification (Nikaido, 1970).

In addition to the O antigenic LPS, at least 24 distinct antigens have been identified in the OM of E. coli, most of which are proteins (Smyth et al. 1978).

The ratio of protein to LPS on the outer surface of enteric bacteria is approx. 3:2 (van Alphen et al. 1977).

A high proportion of the total OM protein content can generally be accounted for by 3 to 5 "major" proteins (Lugtenberg & van Alphen, 1983), prominent amongst which are the porins (Nikaido & Vaara, 1985).

OM proteins are usually identified and defined in

terms of their mobility in SDS-PAGE after Coomassie blue or silver staining of OM preparations and have molecular mass ranging from 30-50 kDa (Benz, 1988). Most, but not all, porins are organized in the outer membrane as trimers of three identical subunits (Angus & Hancock, 1983; Palva & Randall, 1978 and Palva & Westerman, 1979).

The OM proteins are named after the genes that encode them (usually as Omp proteins). The OmpC, OmpF and PhoE proteins of E. coli are "porin" proteins (Nakae, 1976), and allow the diffusion of small hydrophilic solutes (within a limited size range) across the membrane. Substantial amino-acid sequence homology between porin proteins has been demonstrated (Mizuno et al. 1983).

Strong interactions between porin proteins and LPS (Poxton et al. 1985) or peptidoglycan (Lugtenberg & van Alphen, 1983) have been reported which can result in the isolation of detergent-resistant complexes from disrupted OM preparations.

While many of the major OM proteins are constitutively expressed, their production and relative proportions can be modulated by growth conditions (Benz, 1988). The OmpF porin of E. coli for example, is greatly reduced in media of high osmolarity (Kawaiji et al. 1979), while other OM proteins are induced by specific nutrient depletion e.g. PhoE, an anion-selective porin, is produced in E. coli and other Gram-negative bacteria under phosphate limitation (Poole & Hancock, 1986 and van der Ley et al. 1987). Synthesis of other OM proteins is induced in the presence of specific nutrients e.g. the maltose-specific porin, the LamB protein (also a bacteriophage receptor) in response to the presence of maltose (Palva, 1978).

Unlike plasma membranes there appear to be relatively few enzymes localized in the OM, with phospholipase A₁ and proteases being characteristic OM enzymes (Salton, 1987).

The space between the inner and outer membranes that contains the peptidoglycan is called the periplasm. The periplasm is iso-osmotic with the cytoplasm (Stock et al.

1977) and contains a variety of proteins (Beacham, 1979) in addition to the peptidoglycan layer. The main constituent of the periplasm, however, is a gel of highly hydrated peptidoglycan with a low degree of cross-linkage (Hobot et al. 1984).

The Glycocalyx: The glycocalyx is defined as being any polysaccharide containing component outside the cell wall (Costerton et al. 1981) and is an almost universal component of bacterial cells in nature but is frequently lost in laboratory culture. The glycocalyx probably acts as a barrier to the many antibacterial agents found in both natural and pathogenic environments (e.g. bacteriophages, Abs, phagocytes) and has implications in both animal (Costerton et al. 1981) and plant (Chatterjee & Vidaver, 1986) pathology.

Glycocalyxes are subdivided into two types:

- 1). S-layers composed of a regular array of glycoprotein subunits at the cell surface (Sleytr & Messner, 1983);
- 2). Capsules (exopolysaccharides EPS) composed of a fibrous matrix at the cell surface that may vary in thickness and may either be "integral" (i.e. normally associated with the cell surface) or "peripheral" (i.e. may either remain associated with the cell or shed into the surroundings).

Whereas S-layers are generally composed of a single, homogenous, high molecular weight (40-200 kDa), polypeptide species sometimes containing carbohydrate(s) (Sleytr & Messner, 1983), capsules may either be simple homopolymers or very complex heteropolymers of a wide variety of monosaccharides (Sutherland, 1977)

Two types of surface appendages may be present in bacteria; pili, (also called fimbriae) (Paranchych & Frost, 1988) and flagella (Doetsch & Sjoblad, 1980), which although differing in both function and overall form, share many common structural features. Both originate from the cell membrane and extend outward through the wall to a distance of up to ten times the diameter of the wall. Flagella and pili are made up of specific proteins known as

flagellins and pilins respectively; the protein subunits of which have molecular weights of 17 to 50 kDa.

Whereas pili are involved in the adhesion of bacteria to surfaces, flagella are responsible for motility in bacteria.

Flagella have a role as virulence factor in some animal pathogens (e.g. Vibrio cholerae Camphylobacter jejuni, where the bacterium is propelled through the mucus lining the small intestine), but in most cases there is no correlation in plant pathogens (Chatterjee & Vidaver, 1986).

Likewise, pili are considered virulence factors in animal pathogens (Elwell & Shipley, 1980), and despite several reports of their presence in plant pathogens (e.g. Stemmer & Sequeira 1981, and Fuerst & Hayward, 1969), their role in pathogenicity remains unclear.

AIMS OF THIS PROJECT

The primary goal of this project was to develop sensitive and specific immunoassays for the detection of P. syzygii in its host (the clove tree - Syzygium aromaticum) and insect vectors (Hindola fulva and Hindola striata) based on the use of specific monoclonal antibodies (mAbs).

When this project began, the taxonomic designation of P. syzygii was unclear and had been further confused by the discovery of a close serological relationship with P. solanacearum. The basis for this relationship was unknown and it was envisaged that mAbs could be used to both clarify this, and to further investigate the serology of P. syzygii.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1. Bacterial Cultures and Test Material.

Bacterial cultures: Bacterial cultures were obtained either from the Clove Diseases research project (Bogor, Indonesia) (*P. syzygii* and clove *P. solanacearum* isolates), the National Collection of Plant Pathogenic Bacteria (MAFF, Hatching Green, Harpenden, UK) or Dr. M.J. Davis, University of Florida, Ft. Lauderdale, Florida, U.S.A. (*Xyella* spp.).

Clove wood samples: Samples of clove wood, either field specimens from mature trees showing SD symptoms or from *P. syzygii* inoculated, *P. solanacearum* inoculated, or healthy seedlings from inoculation experiments, were obtained from the cloves project in Bogor.

Inoculation of seedlings (up to two years old) with cultures of field isolated bacteria (4×10^5 to 2×10^6) was performed by needle puncture to the stem as described by Hunt et al., (1987). Seedlings exhibiting symptoms of bacterial infection were used for the preparation of wood samples.

Root, stem, and twig samples were supplied, either as thin sections (50 μ m) or 1-5 x 0.5-1 cm portions.

Samples were either collected fresh (for work done in Indonesia), or sent to the UK preserved in acetone (thin sections) or PBS + 0.02% sodium azide (wood portions).

Insect vector samples: Samples of *Hindola striata* and *H. fulva* were supplied by Dr. C. Lomer and Mr B. Stride (Cloves Project), either preserved in acetone or ethanol, or desiccated over silica gel (2-4 weeks). Both adult insects and nymphs were used, Some preliminary work was also done in Bogor using fresh insects.

All insects were collected from clove gardens and were

designated as coming either from a diseased or disease free area.

Storage of bacterial cultures: Cultures were stored on glass beads at -70°C in Casamino acid (+Fe) = (Ca+) (Appendix II) liquid medium containing 10% v/v glycerol as a cryoprotectant.

Culture of bacteria: bacteria were grown either in liquid broth or on the surface of agar plates.

5ml of CA+ broth was inoculated with either P. syzygii or P. solanacearum on a glass bead.

After incubation at 28°C for 40 hours (P. solanacearum) or 70 hours (P. syzygii) the broth was used to inoculate 100ml of CA+ broth which was incubated on an orbital shaker (180 revolutions/minute) at 28°C.

Glass beads (one per plate) were used to inoculate CA+ medium agar plates (PW agar (Appendix II) was used for Xyella spp. Inoculated plates were incubated at 28°C. Samples of broth cultures and resuspended agar plate cultures were checked for purity by streaking on CA+ plates.

Identity of cultures was verified by streaking on to sucrose peptone agar (SPA) (Appendix II); P. syzygii cultures being verified by no or very poor growth on SPA coupled with slow growth on CA+, and P. solanacearum cultures by their characteristic fast growth and copious production of extra-cellular polysaccharide when grown on SPA medium.

Harvesting of bacteria: Bacteria were harvested from broth culture by centrifugation (1500 x g for 10 minutes), followed by three washes in sterile dH₂O to remove medium components.

Bacteria not required for immediate use could be stored as dry pellets at -70°C for several months.

Bacteria were harvested from agar when sufficient growth was present but before viability was lost (1-2 days for P. solanacearum, 7-10 days for P. syzygii). Plates were flooded with 10ml sterile dH₂O and left for 10 minutes

to allow bacteria to begin to detach. A sterile wire loop was used to detach remaining bacteria and the suspension collected in a centrifuge tube. After washing with, and collecting, a further 10ml of dH₂O, the bacteria were pelleted by centrifugation (1500 x g for 10 minutes), and washed for broth cultures.

Sterile conditions and equipment were maintained throughout the harvesting procedure.

Fixation of bacteria: Two fixation methods were used during the project;

1). Heat fixation; harvested, washed bacterial pellets were resuspended in sterile dH₂O (20ml for broth cultures, 5ml for plate cultures) and placed in a steamer (100°C) for one hour. Following determination of the OD₆₅₀, the suspension was pelleted and resuspended in PBS to a volume giving an OD₆₅₀ of 10;

2). Acetone fixation; harvested, washed bacterial pellets were resuspended in acetone (20ml for broth cultures, 5ml for plate cultures) and left at room temperature for 15 minutes. After resuspension in PBS, the suspensions were standardized as in 1). Some cultures (particularly P. syzygii) clumped on resuspension and required sonication (approximately 15-30 seconds) to disperse the clumps.

Suspensions of fixed bacteria can be kept for long periods (months to years) at 4°C in the presence of 0.02% w/v sodium azide.

In addition, samples of unfixed, live bacteria were also prepared for use in assay development and mAb testing. Harvested, washed bacteria were standardized with respect to OD₆₅₀, pelleted and stored at -70°C. When require for use, the pellet was resuspended in the appropriate volume required to give a standardized solution.

2.2. Monoclonal Antibody Production.

Animals: Both rats (Lou/IAP and F344, female, IAP, Babraham) and mice (Balb/c, females, 6 weeks old, Harlan Olac Ltd) were used.

Immunization of animals:

Rats: groups of 2-3 animals were primed with 1×10^8 heat-fixed bacteria (in 200 μ l of FCA) by i.m. injection into both thighs. Boosts were similarly administered (in FIA) at 4-5 week intervals.

Test bleeds (from the tail vein) were taken approximately 14 days after each boost.

Animals selected for mAb production were given a final i.v. boost of Ag (1×10^8 bacteria in 1ml of saline 3 days prior to splenectomy and fusion.

Mice: mice were primed by i.p. injection of either 1×10^8 or 5×10^8 acetone fixed bacteria in 500 μ l of saline and boosted with the same dose 14-26 days later.

Test bleeds (from the tail vein) were taken 7 days after the boost. Animals selected for mAb production were rested for 6 weeks prior to administration of a final i.p. boost 3 or 4 days before splenectomy and fusion.

Titration of test bleeds: Serum was prepared from test bleeds by allowing the blood to clot (1 hour at room temperature) followed by centrifugation 150xg, 10 minutes.

Sera were tested by serial dilution in ELISA (see 2.24) against both P. syzygii (R1) and P. solanacearum (R64) (fixed by the same method as immunogen), using pre-immune serum as a control. Sera were compared with respect to both the specific and cross-reactive titres.

Only animals with a specific titre in excess of 1/1000 were used for mAb production.

Cell culture conditions: All cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with Foetal Calf Serum (FCS) (ranging from 20% v/v (DMEM/20) to 0% (DMEM/0)), glutamine (2mM) and

Penicillin/Streptomycin (100 IU/ml;100 µg/ml). In addition, medium for the culture of mouse hybridomas contained 50 µM 2-mercaptoethanol.

Cells growing in tissue culture plates and in soft agar cloning plates were incubated at 37°C in humid atmosphere of 5% CO₂ in air.

Cultures transferred to flasks or roller bottles were gassed with 5% CO₂ in air and incubated at 37°C with the cap screwed on tight. Further gassing was not required once the cells were growing vigorously.

Preparation of macrophage feeder cells: Peritoneal macrophages (Møs) were prepared by peritoneal lavage of Balb/c mice. 2 x 5 ml of DMEM/20 was injected into the peritoneal cavity using a wide bore hypodermic needle (0.8 mm x 40 mm), after agitation of the peritoneum the medium was withdrawn and made up to the required volume with DMEM/20.

The number of mice used varied from 1 mouse/2 plates (24 or 96 well) in early work, to 1 mouse/6 plates in later work. Møs were not counted as the amount extracted appeared not to vary significantly.

96 well plates were seeded with 100 µl of Mø suspension/well, 24 well plates were seeded with amounts varying from 0-0.5 ml/well. In some cases (rat fusion experiments) the seeded Møs were gamma-irradiated. Irradiation was later found not to be necessary and was not done for the mouse fusions, with only occasional wells being overgrown by fibroblasts. Møs were generally plated 1 day prior to the fusion experiment to allow them to adhere to the plate.

Preparation of myeloma cells: Y3 Ag 1.2.3 (Kappa light chain secretor) and IR983F (non-secretor) myeloma lines were used for the rat fusions, and X63/Ag8/653 for the mouse fusions. Rat myelomas were grown in spinner culture, mouse myelomas in static sealed flasks.

Myelomas were pelleted by centrifugation (100 x g for 5 minutes) and resuspended in 10 ml of DMEM/0 before being

counted using a haemocytometer.

Preparation of spleen cells: Splenectomy was performed on the selected mouse/rat 3-4 days after the final antigen administration. Spleen cells were extracted either by grinding the spleen through a coffee strainer using a syringe plunger (rat fusions) or by perfusing the spleen with 20 ml of DMEM/0 using syringes and fine (0.45 mm x 10 mm) needles (mouse fusions). Spleen cells were pelleted (100 x g for 5 minutes), resuspended in 10 ml of DMEM/0, and counted.

Fusion of myeloma and spleen cells: All stages of the fusion were done in serum free medium (DMEM/0) since the polyethylene glycol (PEG) used as the fusogen would precipitate the serum proteins.

The spleen cells were mixed with myeloma cells to give a ratio of spleen:myeloma of between 4:1 to 10:1 (varied for different fusions) in a 50 ml centrifuge tube and made up to 40 ml with DMEM/0. After centrifugation of the mixture (100 x g for 10 minutes) the supernatant was drained (it is important to completely remove the supernatant to minimise dilution of the PEG) and the pellet broken by gentle tapping of the tube. The tube was then placed in a beaker of water at 37°C and 1 ml of PEG 1500 (50% w/v in 75 mM Hepes (Boehringer Mannheim)) added dropwise over 1 minute whilst stirring. The pellet was stirred for a further 1.5 minutes, followed by the dropwise addition (whilst stirring) of 10 ml of DMEM/0 (prewarmed to 37°C). The mixture was made up to 25 ml and centrifuged (100 x g for 5 minutes). The resulting pellet was resuspended in the required volume of selective medium (Appendix II) (DMEM/20 + HAT in rat fusions, DMEM/20 + HAZa in mouse fusions) and seeded into the Mø coated plates (Day 0). Plates were incubated at 37°C in an atmosphere of 5% CO₂.

The individual fusion protocols, including details of myeloma line, fusion ratio, plate format, and selective agent, are summarized in Table 2.1.

Table 2.1.

**Summary of the Fusion Protocols for Individual Rat and
Mouse Based Fusions**

<u>Fusion Number</u>	<u>Myeloma Line</u>	<u>Fusion Ratio</u>	<u>Plate Format</u>	<u>Selective Agent</u>
SD1	Y3Ag1.2.3.	4:1	24	HAT
SD2	IR983F	4:1	24	HAT
SD5	X63/Ag8/653	4:1	96	HAza
SD6	X63/Ag8/653	5:1	96	HAza
SD7	X63/Ag8/653	5:1	96	HAza
PS1	Y3Ag1.2.3.	4:1	24	HAT
PS2	IR983F	4:1	24	HAT
PS3	X63/Ag8/653	10:1	96	HAza

Fusions SD1, SD2, PS1 and PS2 were rat based

Fusions SD5, SD6, SD7 and PS3 were mouse based

Maintenance and screening of hybridomas: From day 7 post fusion onwards the plates were examined both macroscopically (for yellowing of the medium by growing hybridomas) and microscopically to identify wells containing growing hybridomas.

Once sufficient colonies had been observed (generally between days 10 and 12), cultures were fed by removal of medium and replacement with an equal volume of DMEM/20 + HT (for HAT fusions) or DMEM/20 + H (for Haza fusions). Feeding also serves to remove any residual antibody secreted into the medium by unfused spleen cells.

Supernatant samples were taken from growth positive

wells (0.5 ml from 24 well plates, 100 μ l from 96 well plates) and screened against R1 and R64 by ELISA (fusions SD6 and SD7 were also screened against R6 (P. syzygii)). Screening was often repeated at day 21 to test slower growing hybridomas.

Cloning: Hybridomas identified by ELISA as producing antibody of interest were cloned as soon as possible, either by soft agar cloning (Köhler and Milstein, 1976) (rat 24 well fusions) or by limiting dilution (mouse 96 well fusions).

For soft agar cloning, cells were resuspended directly from the well and serially diluted in 1 ml of DMEM/20 (five dilutions prepared). An equal volume of cloning agar (0.5% w/v, at 45°C) (Appendix II) was added and the mixture poured onto a pre-poured cloning agar base in a petri-dish. The dishes were incubated (at 37°C in an atmosphere of 5% CO₂) for 7 days. 24 colonies were picked from the plate giving the best separation of colonies, each being transferred to 1 ml of DMEM/20 in a 24 well plate.

For limiting dilution cloning, cells were resuspended in the well, counted and diluted to give 5 cells/ml (mouse fusions), and 100 μ l aliquots of each dilution were plated over 48 wells of a 96 well plate that had previously been seeded with MøS.

Wells were examined (as before) for growth (after 7 days in 24 well plates and 14 days in 96 well plates) and screened for Ab production. Any wells containing more than one hybridoma colony were considered not to be cloned and were not tested.

Expansion of selected hybridomas: The three (to cover for any losses at this stage) best clones from each line were selected for expansion.

Clones in 24 well plates were split into two further wells and, after 2-3 days (depending on growth rate) one of the three was transferred to a 15cm² flask in 5 ml of DMEM/20. The flask was gassed with 5% CO₂ in air, sealed and incubated at 37°C. Flasks were made up to 15 ml once

the cells were growing well (usually after 2-7 days). The other two clones were discontinued after four samples of each had been frozen.

Mouse clones were transferred directly from 96 well plates to 10 ml of DMEM/20 in 15 cm² flasks either seeded with MøS (1 mouse/6 flasks) or supplemented with 10% fibroblast conditioned medium (DMEM/20 conditioned by the presence of MRC5 fibroblasts for 24 hours). Cells reached confluency in 7-10 days. This method of expansion was 100% successful thus eliminating the need to expand more than one clone of each line.

Cell freezing: Aliquots of selected lines were frozen at all stages of the procedure. Cells were frozen by pelleting and resuspension in freezing medium (10% v/v DMSO in FCS). 0.5 ml aliquots were dispensed into freezing vials (Nunc) and cooled slowly (by placing in a -70°C freezer) for 24 hrs before being stored in liquid nitrogen (-196°C). At least 4 vials of each uncloned and cloned line was frozen.

Final selection and bulk culture of hybridomas: Supernatant from the 15 ml flask cultures was used for screening against a large panel of bacteria. Hybridomas thus identified as producing useful antibody were bulked up to 1 litre roller bottle cultures by progressive doubling of the medium volume as required by the growth rate of the cells. Generally cultures could be expanded from 15 ml to 1 litre in 10-12 days. The FCS concentration was progressively reduced to 2.5% v/v. The supernatant was harvested by centrifugation 7-10 days after reaching 1 litre. The pelleted cells were returned to the roller bottle, resuspended in 1 litre of DMEM/0 and reincubated for 2-3 days or until the cells died. Not all hybridoma lines survived under such conditions, but those that did produced similar quantities of Ab to when grown in serum containing medium.

Concentration and purification of mAbs: mAbs were initially concentrated from culture supernatant (after

removal of cells by centrifugation) by ultrafiltration using a combination of two ultrafiltration devices: an Amicon CH2 spiral-wound membrane ultrafiltration system capable of concentrating any volume above 200 ml down to 70-100 ml, and an Amicon 8200 stirred ultrafiltration cell capable of concentrating 20-200 ml down to 15-20 ml. 30 000 MW cutoff membranes were used in both devices.

Concentrated mAb (in 15-20 ml) was further concentrated and partially purified by ammonium sulphate precipitation. Saturated ammonium sulphate (Appendix II) was added dropwise over approximately 15 minutes (at room temperature with stirring) to the mAb solution to give a final concentration of 40% v/v, followed by stirring for one hour after which the precipitate was pelleted by centrifugation (1500 x g for 15 minutes). The pellet was dissolved in 1 ml of PBS, and dialysed against 3 x 5 litre of PBS.

The mAbs were stored at -20°C, under which conditions no loss of activity was seen.

Isotype determination of mAbs: The heavy and light chain class of the rat mAbs was determined either by radioactive incorporation (Galfré & Milstein, 1981) (SD1/SD2 and PS1/PS2 mAbs) or by immunodiffusion using a Serotec typing kit (SD3 and SD4 mAbs).

i). Radioactive incorporation:

a). 4 ml of culture from a 15 cm² flask was transferred to a 10 ml "push-on cap" tube;

b). the tube was centrifuged, and the cells resuspended in 200 µl of "incorporation medium" (containing 1 µCi of ¹⁴C-lysine), and incubated overnight;

c). the cells were pelleted, and the supernatant mixed with 200 µl of SDS-PAGE sample buffer;

d). 10 µl samples were separated on 12% SDS-PAGE mini-gels, a Y3 supernatant sample was included as a Kappa light chain standard, IgG & IgM mAbs were also included as standards:

e). gels were autoradiographed (80 hour exposure);

f). developed autoradiographs were examined to determine the heavy and light chain isotypes.

ii). Immunodiffusion:

10 μ l of culture supernatant was tested against 10 μ l each of sheep anti-rat IgM, IgG1, IgG2a, IgG2b, IgG2c, and IgA subclass specific antisera. Precipitin bands were usually visible after 1-2 days incubation of the immunodiffusion plates (at room temperature in a damp box).

The immunoglobulin heavy chain class and subclass, and the light chain isotype of the mouse mAbs was determined by ELISA (using the standard protocol (see Chapter 2.24.) except where indicated):

a). 9 microtitre plate wells were coated with either R1 or R64 (depending on the mAb to be tested);

b). mAb was added to each well and incubated;

c). one of each of the eight typing sera (rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, kappa and lambda light chains (Bio-Rad)) was added to 8 of the wells;

d). anti-rabbit IgG (H+L) peroxidase conjugate (1/5000) was added to each well;

e). the absorbance was read after addition of substrate and stopping of the reaction.

2.3. SDS-PAGE and Immunoblotting.

Sample preparation: live bacteria were harvested from agar plates and standardized as previously described.

To prepare bacterial protein samples 5×10^8 bacteria were taken from the stock suspension, pelleted (by centrifugation at $1500 \times g$ for 5 minutes), resuspended in 1 ml of SDS-PAGE sample buffer (Appendix II), heated at 100°C for 5 minutes, allowed to cool, and centrifuged at $1500 \times g$ for 10 minutes (to remove insoluble matter that may cause streaking in the gel pattern).

Bacterial LPS samples were prepared by the method of Hitchcock and Brown (1983). 1.5×10^9 bacteria were pelleted

from stock solution, resuspended in 50 μ l of SDS-PAGE sample buffer, heated at 100°C for 5 minutes and allowed to cool. 25 μ l of proteinase K (Protease type XI, Sigma) in 10 μ l of sample buffer was added and the mixture incubated at 60°C for one hour followed by centrifugation at 4000 x g for 10 minutes.

Protein molecular weight standards were prepared by dissolving a mixture of myosin (1.7 mg/ml; 205 kDa), β -galactosidase (1 mg/ml; 116 kDa), phosphorylase b (1 mg/ml; 97.4 kDa), bovine serum albumin (1.5 mg/ml; 66 kDa), ovalbumin (1.5 mg/ml; 45 kDa), glyceraldehyde-3-phosphate dehydrogenase (1 mg/ml; 36 kDa), carbonic anhydrase (1 mg/ml; 29 kDa), inactivated trypsinogen (2 mg/ml; 24 kDa), trypsin inhibitor (2.5 mg/ml; 20.1 kDa), and α -lactalbumin (1 mg/ml; 14.2 kDa) in SDS-PAGE sample buffer and heating at 100°C for 5 minutes.

SDS-PAGE: SDS-PAGE was performed on 5-16% gradient gels (Appendix II) using a Bio-Rad Protean II gel apparatus. 20 μ l (protein preparations) or 10 μ l (LPS preparations and molecular weight standards) samples were loaded into each track. 25 mA/gel (constant current) was applied until the bromophenol blue tracking dye in the sample buffer entered the separating gel, when the current was increased to 35 mA/gel. When gels were to be immunoblotted, 5 μ l of 0.25% w/v methyl green (in 10% v/v glycerol) was added to each track. The methyl green is transferred to the nitrocellulose, thus acting as a track marker.

Gels for LPS samples were prepared as for protein gels except that SDS was omitted from the stacking and separating gels.

Staining gels for protein: gels were washed briefly in distilled water (dH_2O) to remove excess SDS, and then immersed in a solution of 0.1% w/v Coomassie blue (Appendix II) for 2 hours. Gels were destained in several changes of destain solution until the protein pattern was clearly visible.

Staining gels for LPS: LPS was identified using the silver stain of Thompson (Hancock and Poxton, 1988) (Appendix II). Digestion of protein was confirmed by staining of gels with Coomassie blue.

Immunoblotting: Both protein and LPS gels were electroblotted using a modification of the method of Towbin et al. (1979), the major difference being the use of a MOPS/sodium acetate/ethanol transfer buffer (Appendix II) which had been observed to facilitate better transfer and to allow the use of higher voltages than tris-glycine buffer (author's unpublished observations).

After washing (2 x 5 minutes in aqueous transfer buffer) to remove excess SDS, gels were electroblotted onto nitrocellulose (0.2 μ m pore size) for 1.5 hours at 180 volts (the current staying constant at 0.35 A or 0.47 A for one or two gels respectively) in a Bio Rad Trans-Blot cell. An aluminium cooling coil recirculating tap water was required to prevent an increase in temperature in the cell.

Upon completion of transfer the nitrocellulose was cut into strips corresponding to the gel tracks. Tracks containing MW standards were stained in 0.5% w/v amido black (Appendix II) and then destained until the bands were clearly visible. Tracks for immunoprobng were immersed in 5% w/v dried milk powder in PBS (5% DMP) (to block remaining protein binding sites) either at 37°C (1 hour) or 4°C (overnight). Gel length was measured to allow calculation of molecular weights.

Immunoprobng of nitrocellulose blots: blots were incubated with mAb or pAb by sandwiching the nitrocellulose between two sheets of filter paper (Whatman no. 1) soaked in Ab solution (in 5% DMP in PBS). The use of Abs at twice their ELISA working dilution was generally found to give good results. The sandwich was clamped between two perspex plates, and incubated in a damp box (37°C for 2 hours). This method is very economical with Ab, requiring approximately 1 ml of Ab solution per cm width of nitrocellulose. After incubation the nitrocellulose was

washed in PBS (3 x 5 minutes) before being immersed in 50 ml of a 1/500 dilution of HRP labelled anti-species IgG (H+L) conjugate (anti-rat or -mouse as appropriate) and incubated at 37°C for 2 hours. After washing as before the nitrocellulose was incubated (at room temperature for 30 minutes) in HRP substrate solution (Appendix II), the reaction being stopped by several rinses in dH₂O.

Determination of the molecular weight of immunodetected bands: the amido black stained MW standards were used to construct a standard curve by plotting $\log_{10} \%T$ against $\log_{10} MW$, where $\log_{10} \%T = \log_{10} (\text{distance travelled by protein} \times \delta 1\% + \text{initial acrylamide concentration})$, and $\delta 1\% = 1/\text{gel length} \times \text{range of acrylamide concentration}$.

The molecular weight of immunodetected proteins was calculated from the standard curve and expressed as kilodaltons (kDa).

2.4. Immunoassays

The assays described below are the basic protocols, variations in sample application and solid phase are described in the relevant Results sections.

Direct Ag binding ELISA (DAB-ELISA): DAB-ELISA was performed using 96 well polystyrene microtitre plates (Nunc). All reagents were used in 100 μ l aliquots unless otherwise stated.

Wells were coated with bacterial suspensions of known bacterial numbers (prepared from stock suspensions at $OD_{650} = 10$) by incubation at either 37°C (1 hour) or 4°C (overnight) in PBS. Unbound material was removed by inversion of the plates followed by washing in three changes of PBSt (3 minutes each). The plates were dried by gentle tapping onto tissue paper on completion of washing. First Ab was applied at the appropriate dilution in a suspension of 5%w/v dried skimmed milk powder (St. Ivel) in PBSt (5%DMPT) and incubated for one hour at 37°C.

Second Ab (HRP conjugated sheep anti-rat IgG (H+L) or sheep anti mouse IgG(H+L) (The Binding Site Ltd) as appropriate) was applied at 1/1000 dilution in 5%DMPT and incubated for $\frac{1}{2}$ hour at 37°C. Between the addition of each reactant the plates were washed as before.

Bound second Ab was detected by the addition of TMB solution (Appendix II). Plates were incubated at room temperature until sufficient colour development occurred (5-15 minutes) the reaction being stopped by the addition of 25 μ l/well of 3M H_2SO_4 . Results were quantified by reading the absorbence at 450nm (A450) (including a turbidity correction of 650nm) using a microtitre plate reader (Flow Laboratories Ltd) blanked against wells containing substrate only.

Duplicate wells were used for all samples. All assays also included appropriate positive controls (i.e. known positive Ab) and negative controls i.e. Ag and substrate only; Ag, 2nd Ab, and substrate only; 2nd Ab and substrate only; and pre-immune serum (or non-reacting mAb).

Double Ab sandwich ELISA (DAS-ELISA): Trapping Ab solution (in carbonate buffer pH9,2) was coated to microtitre plates by incubation for 2 hours at 37°C. Following washing, unoccupied binding sites were blocked by incubation in 5%DMPT for 1 hour at 37°C. The assay then proceeded as described for the DAB-ELISA.

Control wells where bacteria were omitted, were used to blank the plate reader.

Dot immunobinding assay (DIA): Bacterial suspensions in PBS (prepared from stock suspension as for ELISA) were applied to sheets of nitrocellulose membrane (Schleicher and Schuell, 0.2 μ m pore size) prewetted in PBS, in volumes up to 250 μ l using a Bio-Dot apparatus (Bio Rad Ltd). Following washing by immersion in three changes of PBS, remaining binding sites were blocked by incubation of the membrane in 5%DMP in PBS (5%DMP) for $\frac{1}{2}$ hour at room temperature. Incubation with 1st Ab was by the filter paper sandwich method as used for immunoblotting for 2

hours at 37°C. Incubation with 2nd Ab (HRP conjugate etc. as for ELISA, at 1/1000), for 1 hour at 37°C, was carried out by either immersion of the membrane in 2nd Ab solution (in 5%DMP) or by the filter paper method. Between the addition of each reactant the membrane was washed as before. Bound 2nd Ab was detected by incubation of the membrane in substrate solution (4CN - Appendix II) until sufficient colour development occurred (15-30 minutes).

Immunofluorescence assay (IFA): IFA was performed using multiwell microscope slides (Dynatech). All reagents (unless otherwise stated) were applied in 10 µl aliquots in 5%DMP and incubated for 15 minutes at 37°C in a damp box.

Bacterial suspensions (in PBS) were applied to slide wells and dried at 40°C followed by fixation by immersion in acetone (5 minutes). The slides were washed by immersion in PBS (3 minutes) and dried. 1st Ab was applied at the appropriate dilution. 2nd Ab was either anti-mouse IgG(H+L) - FITC(1/50) or anti-mouse IgG(H+L) R-Pe (1/10). Between the addition of each reactant slides were washed as before. 5 µl of phosphate buffered glycerol (Appendix II) was added to each well and the slide was covered with a cover-slip. Slides were examined for fluorescence using a Leitz Orthoplan epifluorescence microscope at 495nm (FITC & RPE) and 555nm (RPE).

Slide agglutination: 50 µl of bacterial suspension in PBS was mixed with 50 µl of Ab in PBS on a microscope slide. The slide was examined for agglutination (as indicated by clumping of the cells) for up to 1 hour afterwards.

Bacteria were also tested against PBS in place of Ab to test for autoagglutination.

Extraction of bacteria from clove wood by elution with potassium hydroxide (KOH): 1-2cm portions of wood were cut and placed either in 50 µl of PBS on a glass slide (for IFA), in 200 µl of PBS in the well of a 48 well tissue culture plate (for ELISA), or on a pre-wetted (with PBS) sheet of nitrocellulose placed over a saturated filter

paper pad (for DIA). One drop (approximately 50 μ l) of 0.1M KOH was applied to the top of the wood portion and the bacteria allowed to elute for 15 minutes.

2.5. Purification of Rabbit IgG by Protein A Affinity Chromatography.

5 g of Protein A-sepharose CL-4B (Pharmacia) freeze dried powder was swollen for 15 minutes in 50 ml PBS followed by washing on a sintered glass filter (G3) with 1 litre of PBS. The washed gel was packed into a glass chromatography column.

1 ml of rabbit anti- P. syzygii serum was loaded onto the column. Unbound material was eluted with 100 ml of PBS. Elution of bound IgG was with 3M potassium isothiocyanate. A flow rate of 50 ml buffer/hour was used, with eluted material being monitored at 280 nm.

1 ml samples of the IgG peak were collected, and, after dialysis against PBS, tested by ELISA for anti- P. syzygii activity.

CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES

3.1. INTRODUCTION

The most important factor determining the specificity and sensitivity of any immunoassay is the quality of the antibody reagent used. PAbs produced against P. syzygii (Eden-Green, 1987c) were of poor quality in this respect in that they cross-reacted with P. solanacearum, and were of low titre after cross-absorbition. The hybridoma technique, in allowing the selection of mAbs of the required specificity and sensitivity, offered the methodology by which a reagent of the required quality could be obtained.

Because the assays to be developed by this project were required to detect bacteria in both plant and insect material, under either field or less than ideal laboratory conditions, the development of assays involving the minimum of sample preparation was an important consideration.

Assays utilizing whole bacterial cells, either in vivo or in vitro, whilst allowing for minimal sample preparation and processing, require the use of mAbs directed against cell surface Ags.

Bacteria can be regarded as complex Ags (Challacombe, 1988), a variety of molecules being expressed on their surface. Immunization with whole bacterial cells would therefore be expected to result in an antiserum containing Abs reactive with both specific and cross-reactive determinants.

The specificity of pAbs may be improved by the use of purified specific bacterial components as immunogen. This requires a method for the identification and purification of such components, and restricts the response to those molecules that can be identified by the method employed.

The hybridoma technique (coupled with the appropriate screening assay), by selecting for specificity at the level of Ab, allows the use of whole bacterial cells as immunogen. Holme & Gustafsson (1985) obtained group- and type-specific mAbs reactive with LPS after immunization with whole cells of Vibrio cholerae, whereas the use of purified LPS as immunogen produced mainly cross-reactive mAbs directed towards the LPS core region; thus demonstrating that the use of whole bacterial cells may be preferable in some cases.

Prior to the start of this project, P. syzygii had been poorly characterized, and the basis of its serological relationship with P. solanacearum was unknown. The use of whole bacterial cells as immunogen was chosen as being the simplest and most effective approach for the production of specific mAbs to a variety (if present) of epitopes. Potentially specific mAbs were identified by screening hybridoma supernatants against both P. syzygii and P. solanacearum, prior to more detailed characterization.

A simple fixation method that could be applied, if necessary, to test samples, as well as allowing the generation of specific mAbs when applied to immunogen preparations, was also needed.

The use of live bacterial cells for immunization had been found to be unsatisfactory by several investigators. Elrod (1941, cited by Schaad (1979)) was unable to distinguish between strains of Erwinia carotovora, E. atroseptica, and E. aroideae using antiserum produced to live cells and suggested that the cross-reactivity was due to common flagellar Ags.

The cross reactivity of antiserum produced to live cells of Xanthomonas with other bacterial species was found to be due to the mucoid polysaccharide on the cell surface (Elrod and Braun (1947a and 1947b), cited by Schaad (1979)). In addition immunisation of rabbits with live P. syzygii cells caused distress and sometimes death of the animal (Dr S.J. Eden-Green; personal communication)

possibly as a result of the toxicity of the LPS.

The use of heat fixed bacteria was recommended for the production of specific antisera (Schaad 1979) but was not successful when applied to P. syzygii in this project. When Thaveechai and Schaad (1984) compared antisera raised to formaldehyde and glutaraldehyde fixed cells of Xanthomonas campestris as well as to trichloroacetic acid extracts and ribosomal extracts in immunofluorescence, they found that none of the antisera were able to differentiate X. campestris from other Xanthomonads.

Antisera produced in rabbits to both heat and glutaraldehyde fixed P. syzygii cross-reacted with P. solanacearum (Eden-Green 1987c).

Cornett et al. (1985) found that specific mAbs produced against unfixed cells of Fusobacterium nucleatum did not react with formaldehyde fixed cells, and suggested that the fixation destroyed or chemically altered the determinant involved. This suggests that despite formaldehyde fixation having been widely and successfully used for the preparation of bacteria for both mAb and pAb (Alvarez & Lou, 1985), its use may be inappropriate for the production of mAbs for discriminating two closely related organisms if the antigenicity of certain molecules is destroyed. Problems may also occur if mAbs produced to chemically altered determinants are required for use with unfixed organisms. In addition the use of hazardous chemicals such as formaldehyde in a diagnostic test is undesirable, particularly if it may be used by non-scientific staff.

In the absence of further comparative data on bacterial immunogen preparation acetone fixed bacteria (Dr I. Tiffin; personal communication) were used as immunogens in the mouse based work.

It is important to use a representative isolate of the species for use as immunogen, coupled with screening of resultant mAbs against a large panel of isolates of the species as well as representatives of other species and

genera, in order to ensure the production and isolation of specific mAbs recognising all (or as many as possible) variants of the species.

The P. syzygii isolate R1 (P. syzygii type strain (Roberts et al., 1990)) and P. solanacearum isolate R64 (a typical clove isolated P. solanacearum biovar 3, shown to be pathogenic to tomato plants (Dr. S. J. Roberts, personal communication)), were chosen as being suitable representatives for use as immunogen and primary screening Ag for mAb production.

Differences in surface Ag expression may occur between bacteria growing in vivo and in vitro and also on different media (Hancock & Poxton, 1988). Unfortunately, the growth of bacteria for immunization and screening in vivo was not possible because of the unavailability in the UK of suitable host plants for the inoculation of characterized isolates of P. syzygii.

In order to minimize potential antigenic variation between isolates, all bacteria (where possible) used for immunization and screening were cultured using the same medium (casamino acids medium, supplemented with iron (CA+)). Also, since bacteria may absorb Ags from the culture medium (Challacombe, 1988), the use of different media for P. syzygii and P. solanacearum may have led to erroneous results during mAb screening. To guard against the possibility of differential uptake of medium Ags by different species, all mAbs selected for further characterization were screened for reactivity with CA+ medium.

Monoclonal antibodies were produced by the method of Galfré and Milstein (1981) with some modifications.

MABs selected for further study were concentrated and partially purified from bulk culture before being characterized with respect to their isotype, working dilution (by titration in ELISA), limits of detection, antigen fixation requirements, and the bacterial component with which they were reactive.

3.2. RESULTS

3.2.1 Isolation of Monoclonal Antibodies

Rat mAbs: Despite several fusions being performed, no specific mAbs against either P. syzygii or P. solanacearum were isolated. Five mABs (all IgM, with kappa light chains); SD1.2, SD2.1, SD2.3, (from P. syzygii immunized rats), PS1.3 and PS2.3 (from P. solanacearum immunized rats) were retained for further study. The results of the first two fusions for each Ag (SD1, SD2, PS1, and PS2) are summarized in Table 3.1.

Mouse mAbs: Four mouse based fusions were performed, three using P. syzygii immunized animals (SD5 (immunized with 1×10^8 bacteria/injection), SD6 (1×10^8 bacteria/injection) and SD7 (5×10^8 bacteria/injection)) and one using a P. solanacearum immunized animal (PS3 (1×10^8 bacteria/injection)).

From these fusions, ten P. syzygii specific mAbs were isolated: (SD5.1 (IgG2a), SD5.2 (IgG1), SD5.3 (IgG3), SD5.4 (IgG2a), SD6.1 (IgG1), SD6.2 (IgG2b), SD6.3 (IgG3), SD7.1 (IgG3), SD7.2 (IgG1) and SD7.3 (IgG1)), one P. solanacearum specific mAb (PS3.1 (IgG1)) and two cross-reactive mAbs (SD7.4 (IgM) and PS3.2 (IgG (subclass not determined)). All mAbs had kappa light chains.

The fusion results are summarized in Table 3.1.

3.2.2 Titration of the mAbs and Determination of their Working Dilution in DAB-ELISA.

The working dilution of the partially purified and concentrated mAbs from fusions SD5, SD6, SD7 and PS3 were determined by serial dilution against their respective Ag (either R1 or R64 at $OD_{650} = 0.1$) by DAB-ELISA. The dilution series were started at the dilution equivalent to neat culture supernatant for each mAb.

The working dilutions, determined as the first dilution in the descending portion of the curve, were: SD5.1 (1/125), SD5.2 (1/75), SD5.3 (1/240), SD5.4 (1/280), SD6.1 (1/1000), SD6.2 (1/100), SD6.3 (1/2400), SD7.1 (1/1200), SD7.2 (1/1200), SD7.3 (1/450), SD7.4 (1/120), and PS3.1 (1/4000).

Table 3.1

Summary of Fusion Results

The results of the individual fusions with respect to the success of the fusion and the mAbs produced from it are summarized in Table 3.1.

KEY

^a : number of wells positive for growth (i.e. containing one or more growing hybridomas)/number of wells seeded with fusion mixture;

^b : number of hybridomas growing/number of myeloma cells used for fusion;

^c : number of Ab producing wells/number of growth positive wells;

^d : number of supernatants containing Ab specific for homologous Ag;

^e : number of supernatants containing cross-reacting Ab;

^f : specific mAb producing clones isolated and selected for further study after cloning and screening against the full bacterial test panel;

^g : cross-reactive mAb producing clones isolated and selected for further study after cloning and screening against the full bacterial test panel;

^h : titre of the serum from the final bleed of the animal used for the fusion when tested against both homologous and heterologous Ag;

ⁱ : most of the specific supernatants did not react with a Javan isolate of P. syzygii. Only mAbs reactive with all P. syzygii isolates were selected;

ND : not determined due to the presence of multiple hybridoma colonies in the wells.

Fusion Number	Growth +ve wells ^a	Fusion Efficiency ^b	Ab +ve Wells ^c	Specific Supernatants ^d	Cross-reactive Supernatants ^e	Specific mAbs Isolated ^f	Cross-reactive mAbs Isolated ^g	Final Bleed Titre ^h	
								Homologous	Heterologous
SD1	74/96	ND	18/74	0	18	0	1 (SD1.2)	>1/25600	1/25600
SD2	71/96	ND	18/71	0	18	0	2 (SD2.1; SD2.3)	>1/25600	1/25600
SD5	142/480	0.001%	23/142	6	17	4 (SD5.1; SD5.2; SD5.3; SD5.4)	0	>1/6400	1/1600
SD6	177/480	0.0018%	18/177	11	7	3 ^L (SD6.1; SD6.2; SD6.3)	0	>1/12800	>1/12800
II I SD7	200/480	0.002%	97/200	80	17	3 ^L (SD7.1; SD7.2; SD7.3)	1 (SD7.4)	>1/12800	1/6400
PS1	81/96	ND	67/81	0	67	0	1 (PS1.3)	1/6400	1/6400
PS2	83/96	ND	72/83	0	72	0	1 (PS2.3)	1/6400	1/6400
PS3	101/480	0.002%	42/101	3	39	1 (PS3.1)	1 (PS3.2)	>1/6400	1/3200

The mAbs were used at their working dilutions in all subsequent studies (unless otherwise stated).

A mixture of seven P. syzygii specific mAbs (SD5.4, 6.1, 6.2, 6.3, 7.1, 7.2 and 7.3) designated SD.SM (SDB (P. syzygii) specific mAb mixture) was used in some studies, in which the component mAbs were present at their individual working dilutions.

3.2.3 Determination of the Limits of Detection of the P. syzygii specific mAbs in DAB-ELISA.

The limits of detection (minimum number of bacteria detectable over the threshold value) of the P. syzygii specific mAbs and SD.SM were determined by testing against serial dilutions of bacteria, R1 and R64 (from 1×10^7 - 2×10^4) using the DAB-ELISA.

The threshold value was determined from the maximal A_{450} obtained against R64 (no greater than 0.05, in any case).

The results (summarized in Table 3.2) show that most of the mAbs have similar detection limits. The use of the SD.SM increases the sensitivity of the assay by a factor of two with respect to the best single mAb (SD7.3).

Table 3.2.

The Detection Limits of P. syzygii Specific mAbs

<u>mAb</u>	<u>Detection limit</u>
SD5.4	3.12×10^5
SD6.1	1.56×10^5
SD6.2	3.12×10^5
SD6.3	3.12×10^5
SD7.1	1.25×10^6
SD7.2	3.12×10^5
SD7.3	7.8×10^4
SD.SM	3.9×10^4

The detection limits (minimum number of bacteria resulting in a signal greater than the threshold) of mAbs selected for use in the development of diagnostic assays were calculated from curves obtained in DAB-ELISA.

3.2.4. Determination of the Spectrum of Bacterial Reactivity of the mAbs by DAB-ELISA.

The reactivity of the mouse mAbs towards a wide range of bacterial isolates was assessed by DAB-ELISA. The bacterial test panel included a range of P. syzygii isolates (Table 3.3), both clove and representative race and biovar isolates of P. solanacearum (Table 3.4), and a range of plant pathogenic and other bacteria (Table 3.5). Polyclonal antisera taken from the mice used for fusions SD5 (anti-P. syzygii) and PS3 (anti-P. solanacearum) were also tested against the bacterial panels. The sera were used at 1/2000 and 1/500 respectively.

The threshold for each individual isolate in the panel was determined as twice the absorbance value obtained with normal mouse serum (all values were 0.06 or less).

The results are summarized in Table 3.6.

All of the mAbs were unreactive against CA+ broth (coated to ELISA plates by incubation at 37°C for 2 hrs).

Limited testing of the five rat mAbs showed them to be reactive with all P. syzygii (5 isolates) and P. solanacearum (R64 and all four biovars) isolates tested. Slight reactivity was seen against P. fluorescens and P. cepacia in some cases.

Table 3.3

MAB Test Panel 1 : P. syzygii Isolates

<u>Isolate no.</u>	<u>Isolated From</u>	<u>Location</u>
R1	Clove	W. Sumatra (Solok)
R2	Clove	W. Java (Sukamantri)
R3	Clove	W. Java (Sukamantri)
R4	<u>Hindola fulva</u>	W. Sumatra (Solok)
R5	Clove	W. Java (Sukamantri)
R6	Clove	W. Java (Sukamantri)
R8	<u>Hindola fulva</u>	W. Sumatra (Solok)
R22	Clove	W. Java (Ciomas)
R23	Clove	W. Java ^a
R24	Clove	W. Java (Pasir Madang)
R25	Clove	W. Java (Pasir Madang)
R26	Clove	W. Java (Pasir Madang)
R52	Clove	W. Java (Sukamantri)
R53	Clove	W. Java (Sukamantri)
R54	Clove	W. Java (Sukamantri)
R56	Clove	W. Sumatra (Solok)
R57	Clove	W. Sumatra (Solok)
R58	Clove	W. Sumatra (Solok)
R78	<u>Hindola fulva</u>	W. Sumatra (Solok)
R79	<u>Hindola fulva</u>	W. Sumatra (Solok)
R81	<u>Hindola fulva</u>	W. Sumatra (Solok)
R83	Clove	W. Java (Sukamantri)
R84	Clove	W. Java (Sukamantri)
R85	Clove	Sumatra (Lampung)
R86	Clove	Sumatra (Lampung)
R88	Clove	Sumatra (Lampung)
R89	Clove	Sumatra (Lampung)
R93	Clove	Sumatra (Bengkulu)
R94	Clove	Sumatra (Bengkulu)
R95	Clove	Sumatra (Bengkulu)
R96	Clove	Sumatra (Bengkulu)
R97	Clove	Sumatra (Bengkulu)
R98	Clove	Sumatra (Bengkulu)
R99	Clove	Sumatra (Bengkulu)
R101	Clove	Sumatra (Jambi)
R102	Clove	N. Sumatra
R103	Clove	N. Sumatra
R104	Clove	N. Sumatra
R106	Clove	N. Sumatra
R107	Clove	N. Sumatra
R108	Clove	N. Sumatra
R109	Clove	N. Sumatra
R110	Clove	Sumatra (Bengkulu)

^a - precise origin uncertain

Table 3.4**MAB Test Panel 2 : P. solanacearum Isolates**

<u>Isolate No.</u>	<u>Biovar/Race</u>	<u>Host</u>	<u>Location/Source</u>
R38	I	Tomato	USA (NCPBP 325)
R39	II	Potato	Egypt (NCPBP 909)
R40	III	<u>Physalis</u>	Costa Rica (NCPBP 790)
R41	IV	Ginger	Hawaii (NCPBP 1579)
R12b	I	Clove	Sumatra
R27	IV	Potato	E. Java
R28	ND	Tomato	Sumatra
R45	ND	Clove	Sumatra (NCPBP 3219)
R64	III	Clove	W. Java
R65	III	Clove	W. Java
R124	IV/1	Ginger	Hawaii (NCPBP 1579)
R125	2	Banana	Peru (NCPBP 2315)
R126	3	Potato	Australia (NCPBP 2316)
R127	I	NG	Puerto Rico (NCPBP 1226)
R128	II	Potato	India (NCPBP 1331)
R129	IV	NG	Australia (NCPBP 1400)
R130	III	NG	Mauritius (NCPBP 1484)
R131	III/1	NG	Hawaii (NCPBP 1581)
R132	III	Potato	Fiji (NCPBP 1702)
R133	1	NG	Colombia (NCPBP 2314)
R141	I	Clove	W. Sumatra
R142	II	Clove	W. Java
R143	III	Clove	W. Java

Footnote: Biovars are shown in Roman numerals

Races are shown in Arabic numerals

NCPBP : culture obtained from the National
Collection of Plant Pathogenic Bacteria.

NG : host not given

Table 3.5

MAB Test Panel 3 : Other Bacterial Genera and Species

<u>Isolate no.</u>	<u>Species</u>	<u>Source</u>
R11	<u>Bacillus subtilis</u>	
R36	<u>Xanthomonas campestris</u> <u>pv. campestris</u>	NCPPB 528
R37	<u>Erwinia amylovora</u>	NCPPB 595
R42	<u>Pseudomonas fluorescens</u>	NCPPB 1964
R43	<u>Pseudomonas corrugata</u>	NCPPB 2445
R44	<u>Pseudomonas cepacia</u>	NCPPB 2993
R67	Pierces Disease Bacterium	J. Wells USA
R73	Periwinkle Wilt Bacterium	J. Wells USA
R74	Almond Leaf Scorch	J. Wells USA
R75	Ragweed Stunt Bacterium	J. Wells USA
R76	Mulberry Leaf Scorch	J. Wells USA
R111	<u>Escherichia coli</u>	NCTC* 9001
R120	<u>Clavibacter michiganensis</u>	NCPPB 1109
R121	<u>Erwinia carotovora</u> <u>subsp. atroseptica</u>	NCPPB 549
R122	<u>Flavobacterium</u> spp.	NCPPB 3070
R123	<u>Pseudomonas aeruginosa</u>	NCPPB 1965
R134	<u>Rhodococcus fascians</u>	NCPPB 2551
R135	<u>Serratia proteamaculans</u>	NCPPB 245
R136	<u>Acetobacter pasteurianus</u>	NCPPB 463
R137	<u>Agrobacterium tumefaciens</u>	NCPPB 2437
R144	Banana Blood Disease	S.J. Eden-
R145	Banana Blood Disease	Green from
R146	Banana Blood Disease	S.Sulawesi

* : NCTC = National Collection of Type Cultures.

Table 3.6
Summary of the Reactivity of the mAbs with the Bacterial Test
Panels as Determined by DAB-ELISA

<u>mAb</u>	<u>Bacterial Panel</u>		
	<u>Panel 1^a</u>	<u>Panel 2^b</u>	<u>Panel 3^c</u>
SD5.1	9 (1)	0	3 (3)
SD5.2	22 (8)	5 (2)	3 (3)
SD5.3	24 (7)	7 (1)	3 (3)
SD5.4	35	0	0
SD6.1	35 (1)	0	0
SD6.2	35 (1)	0	0
SD6.3	34	0	0
SD7.1	32 (3)	0	0
SD7.2	35 (1)	0	0
SD7.3	35	0	0
SD7.4	35	23 (2)	5 (2)
PS3.1	0	6	1
PS3.2	13	22 (1)	4 (3)
PAb 1 ^d	35	23 (4)	6 (3)
PAb 2 ^e	35	23	10 (6)

^a - total of 35 P. syzygii isolates in Panel 1

^b - total of 23 P. solanacearum isolates in Panel 2

^c - total of 23 other bacterial isolates in Panel 3

^d - PAb 1 = mouse anti-P. syzygii pAb

^e - PAb 2 = mouse anti-P. solanacearum pAb

The reactivity profile of each mAb (and two polyclonal antisera) with the isolates in the bacterial test panels are summarized.

The results are expressed as the number of isolates within each of the three test panels with which the mAb was reactive. Figures given in brackets represent the number of reactive isolates giving a weak positive reaction (i.e A_{450} less than 0.2).

Of the four mAbs produced in fusion SD5 which were specific for P. syzygii on initial screening, only one (SD5.4) was found to be reactive with all P. syzygii isolates in the full test panel. The other three (SD5.1, SD5.2, and SD5.3) reacted only with isolates from Sumatra (either some or all) and not against Javan isolates (the immunizing strain was Sumatran in origin).

Whereas SD5.4 was completely specific for P. syzygii, SD5.2, and SD5.3 both reacted with a few of the P. solanacearum isolates and SD5.1, SD5.2 and SD5.3 all showed slight reactivity towards E. coli, Rhodococcus fascians and S. proteamaculans.

In order to obtain more mAbs reactive to all isolates, two further fusions were performed (SD6 and SD7) using mice already immunized with R1. While immunization with a Javan isolate might have been a more obvious choice, mice immunized with R1 were ready for use (only requiring a final boost). In addition, isolation of SD5.4 demonstrated that such mAbs could be isolated, and also the same situation may have occurred (only in reverse) if a Javan isolate had been used (i.e. the generation of mAbs specific only for Javan isolates).

In order to reduce the number of mAbs requiring further characterization, the supernatants from fusions SD6 and SD7 were initially screened against R1 (Sumatran), R6 (Javan) and R64, and only those reactive with both R1 and R6 were selected for a further testing.

Fusions SD6 and SD7 both produced three P. syzygii specific mAbs each (SD6.1, .2, and .3; and SD7.1, .2, and .3). A cross-reactive mAb, SD7.4 was also selected for further characterization.

With a few exceptions, all six specific mAbs reacted with all P. syzygii isolates in the test panel.

The cross-reactive mAb SD7.4 was reactive with virtually all P. syzygii and P. solanacearum isolates as well as BDB, P. cepacia and Rhodococcus (weakly).

Fusion PS3 produced several cross-reactive and specific mAbs. Only the test panel results for PS3.1 (specific, reacting with 5/7 P. solanacearum biovar 3 isolates plus one uncharacterized (with respect to biovar)

P. solanacearum isolate and one BDB isolate (R146); and PS3.2 (cross-reactive, reactive with all P. solanacearum isolates (except R65), some P. syzygii isolates, and a few other bacteria)) are shown as those for the other mAbs were variable and did not correlate with any known factors.

The mouse polyclonal antisera both reacted with all P. syzygii and P. solanacearum isolates in the test panel. The anti-P. syzygii serum also reacted quite strongly with all three BDB isolates, and weakly with three others (R111, R134, and R135). The anti-P. solanacearum serum reacted strongly with the three BDB isolates and P. aeruginosa, and weakly with R11, R42, R111, R134, R135, and R136.

3.2.5. The Designation of P. syzygii Serogroups.

Collation of the results obtained with those mAbs reactive with only some of the P. syzygii isolates (i.e. SD5.1, SD5.2, SD5.3, and PS3.2), reveals several different patterns of reactivity of isolates with the mAb panel (see Table 3.7). MAb SD7.3 is also included in the panel as a P. syzygii positive control.

Four distinct serogroups (1, 2, 3, and 4) were revealed by the mAb panel, based on reactivity with SD7.3 (P. syzygii positive "control" mAb) and at least one of the other mAbs. A fifth group, serogroup 5, which contains six isolates from various locations, includes isolates reactive only with SD7.3.

Serogroup 5 may either represent isolates which have lost the Ags reactive with the other mAbs on the panel (possibly during culture), or may contain one or more additional serogroups for which discriminating mAbs were not isolated.

With a few exceptions, there is a good correlation between the serogroup of the isolates (26/35 tested) and their geographical origin. The best correlation is between serogroup 1 and West Sumatran isolates which is mutually exclusive. 5/7 North Sumatran isolates belong to serogroup 2 (the only other serogroup 2 isolate being R53 (West Javan)). 5/8 West Javan (all from Sukamantri and Ciomas)

isolates belong to serogroup 4 (which does not contain isolates from any other region), whereas R24 and R26 (from Pasir Madang in West Java) both belong to serogroup 5; and 5/9 isolates from the adjacent provinces of Lampung and Bengkulu belong to serogroup 3 (which contains only one other isolate, R107, from another region). The remaining isolates either belong to serogroup 5 or having a unique reactivity.

The regions from which the isolates were obtained are indicated on the map of Java and Sumatra in Figure 3.1.

3.2.6. The Use of DAB-ELISA to Compare the Reactivity of the mAbs with Fixed and Unfixed Bacteria

The reactivity of the mouse mAbs and one rat mAb with acetone fixed, heat-fixed, and unfixed bacteria was compared by DAB-ELISA, both to assess the need for fixation of test samples and to examine the effect of the different fixation methods on the epitopes involved. PS3.2 was not tested.

The rat mAb (produced to heat fixed bacteria) gave an equivalent reaction with acetone fixed and unfixed bacteria.

Many of the mAbs produced to acetone fixed bacteria however, did not react with heat fixed bacteria, and a few had reduced reactivity with unfixed bacteria. In addition SD5.3 was reactive towards heat fixed and unfixed P. solanacearum (although to lesser degree than with P. syzygii).

The results are summarized in Table 3.8.

Negative control values (with MAC193 (rat anti-ovine placental lactogen mAb, supplied by Dr G. Butcher; AFRC-IAPGR), for the rat mAb, and pre-immune mouse serum for mouse mAbs) were 0.06 or less against all Ags. To accentuate all negative results, A_{450} values of 0.12 (2 x the maximal negative control value) or less are represented in the table by a "-".

Table 3.7

The serogroup, as derived from the results obtained with the serotyping mAb panel (SD5.1, SD5.2, SD5.3, PS3.2, and SD7.3), of each *P. syzygii* isolate used in the study is indicated. the geographical origin and ecotype group (bracketed, in Roman numerals, under the serogroup heading) of the isolates is also indicated.

KEY:

Origin of isolates:

WS: West Sumatra
NS: North Sumatra
Lam: Lampung
Beng: Bengkulu
WJ(S): West Java (Sukamantri)
WJ(P): West Java (Pasir Madang)

Scoring of Reactivity:

		<u>A₄₅₀</u>
-	:	0 - 0.099
±	:	0.1 - 0.199
+	:	0.2 +

Isolates for which a serogroup could not be assigned are indicated by a "?". The ecotype of some isolates was not determined (ND).

Table 3.7
The Correlation Of P. syzygii Serogroups With The
Geographical Origin Of The Isolates Within Them

<u>REACTIVITY Vs mAbs</u>							
<u>ISOLATE</u>	<u>ORIGIN</u>	<u>SD5.1</u>	<u>SD5.2</u>	<u>SD5.3</u>	<u>P83.2</u>	<u>SD7.3</u>	<u>SEROGROUP</u>
R1	WS	+	+	+	-	+	1 (I)
R4	"	+	+	+	-	+	1 (I)
R8	"	+	+	+	-	+	1 (I)
R56	"	+	+	+	-	+	1 (I)
R57	"	+	+	+	-	+	1 (I)
R58	"	±	+	+	-	+	1 (I)
R78	"	+	+	+	-	+	1 (I)
R79	"	+	+	+	-	+	1 (I)
R81	"	+	+	+	-	+	1 (II)
R102	NS	-	±	+	+	+	2 (I)
R103	"	-	±	±	+	+	2 (I)
R104	"	-	+	+	+	+	2 (I)
R106	"	-	+	+	+	+	2 (I)
R109	"	-	+	+	+	+	2 (I)
R107	"	-	±	±	-	+	3 (II)
R108	"	-	-	-	-	+	5 (II)
R86	Lam	-	+	±	-	+	3 (ND)
R88	"	-	±	±	-	+	3 (II)
R89	"	-	-	-	-	+	5 (I)
R96	Beng	-	+	+	-	+	3 (I)
R99	"	-	±	+	-	+	3 (I)
R110	"	-	±	±	-	+	3 (II)
R93	"	-	-	-	-	+	5 (II)
R94	"	-	-	-	-	+	5 (II)
R95	"	-	-	±	-	+	? (II)
R2	WJ (S)	-	-	-	+	+	4 (II)
R3	"	-	-	-	+	+	4 (II)
R5	"	-	-	-	+	+	4 (II)
R6	"	-	-	-	+	+	4 (II)
R84	"	-	-	-	+	+	4 (II)
R23	"	-	-	±	+	+	? (ND)
R54	"	-	±	-	+	+	? (II)
R53	"	-	±	+	+	+	2 (II)
R24	WJ (P)	-	-	-	-	+	5 (II)
R26	"	-	-	-	-	+	5 (II)

Figure 3.1

Figure 3.1 shows the major regions of the islands of Sumatra and Java.

Where more specific isolation areas are given in the text, these are indicated on the map.

Sukamantri (not shown on the map) is within the vicinity of Bogor.

The regions where a good serogroup correlation was seen are indicated on the map:

Serogroup 1 (West Sumatra):

////

Serogroup 2 (North Sumatra):

////

Serogroup 3 (Lampung and Bengkulu):

////

Serogroup 4 (West Java (Bogor area):

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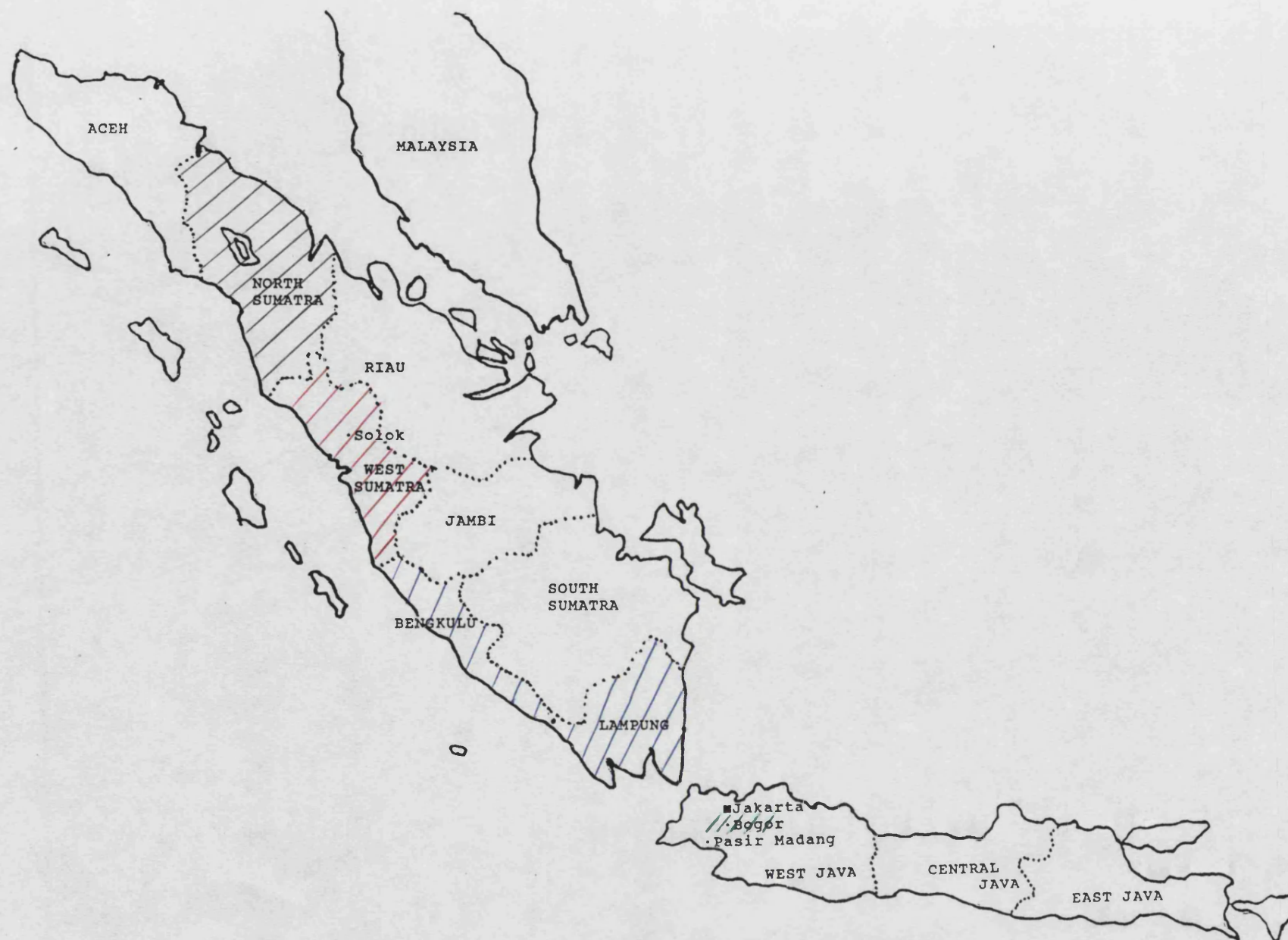


Table 3.8
Reactivity of the mAbs with Acetone Fixed, Heat Fixed, and
Unfixed Bacteria in DAB-ELISA

<u>mAb</u>	<u>A_g^a</u>	<u>A₄₅₀</u>					
		<u>P. syzygii</u>			<u>P. solanacearum</u>		
		<u>Af^b</u>	<u>Hf^c</u>	<u>Uf^d</u>	<u>Af</u>	<u>Hf</u>	<u>Uf</u>
SD2.3	R1/Hf	2.05	2.12	2.07	2.10	2.07	1.93
SD5.1	R1/Af	0.83	-	0.21	-	-	-
SD5.2	R1/Af	0.70	0.72	0.23	-	-	-
SD5.3	R1/Af	1.73	1.62	0.32	-	0.43	0.10
SD5.4	R1/Af	1.56	-	1.75	-	-	-
SD6.1	R1/Af	1.12	-	1.11	-	-	-
SD6.2	R1/Af	1.72	-	2.23	-	-	-
SD6.3	R1/Af	0.64	-	0.76	-	-	-
SD7.1	R1/Af	0.49	-	0.25	-	-	-
SD7.2	R1/Af	1.50	-	2.27	-	-	-
SD7.3	R1/Af	1.34	1.26	1.32	-	-	-
SD7.4	R1/Af	0.59	1.20	1.88	0.79	1.50	0.32
PS3.1	R64/Af	-	-	-	2.21	-	2.05

The reactivity of the mAbs with acetone fixed, heat fixed, and unfixed bacteria (both homologous and heterologous) was determined.

The table shows the results obtained after testing mAbs produced to either heat fixed (Hf) or acetone fixed (Af) bacteria (P. syzygii (R1) and P. solanacearum (R64)) against acetone fixed, heat fixed, and unfixed cells of both P. syzygii and P. solanacearum in DAB-ELISA.

Key: a). Ag : immunizing bacteria and fixation treatment used to produce mAb;
b). Af: Acetone fixed cells;
c). Hf: Heat fixed cells
d). Uf: Unfixed cells;
"-": A₄₅₀ < 0.12.

3.2.7. Partial Characterization of the Bacterial Components Recognised by the mAbs using SDS-PAGE/Immunoblotting

The bacterial components recognised by the mAbs were identified by SDS-PAGE followed by immunoblotting. Both specific and cross-reactive mAbs were tested, to determine the extent and basis of both the similarities and differences between P. syzygii and P. solanacearum.

MABs were tested against R1, R2, R86, R94, R102 (as geographical representatives of P. syzygii), R68, R65, (P. solanacearum clove isolates) R38, R39, R40 and R41 (P. solanacearum biovars 1 - 4).

No reaction was obtained with mAbs SD5.1, SD5.2, SD5.3, and SD7.2.

MABs SD5.4, SD6.1 (Plate 3.1) and SD6.3 (Plate 3.2) all reacted strongly with a single 45 kDa band (three additional bands between 13 and 16 kDa were seen against R1 only with SD6.3). R2, R86 and R102 gave stronger and wider bands than R1, R94 and R96 suggesting the presence of more of the Ag in the former three isolates.

SD7.1 reacted with a band of approximately 76 kDa with isolates R1, R2, R86 and R102 (Plate 3.3). The slightly different mobilities seen with the different isolates was probably due to gel conditions rather than differences in the molecule. No band was seen in the R94 and R96 tracks.

SD7.4 (Plate 3.4) reacted with seven bands from 20 to 24 kDa, the latter being the major band against all P. syzygii and P. solanacearum isolates.

SD6.2 and SD7.3 (Plate 3.5) both produced similar multi-band patterns ranging from approximately 22 kDa to 220 kDa against a uniformly stained background. SD6.2 gave a much weaker reaction than SD7.3.

PS3.1 produced multiple bands from 97 kDa to approximately 220 kDa against R64 and R40 with weaker staining against R39 and R41 (Plate 3.6). No bands were produced against R1 and R38.

PS3.2 reacted with R2, R102, R64, R65 (weakly), R38,

R39, R40, and R41, producing an unusual band pattern that showed variation between the isolates tested (Plate 3.7).

All P. solanacearum isolates (except R65) had a major band at 79 kDa (with R38 and R64 also having a faint band at 177 kDa) followed by a variable series of bands, the most intensely stained portion of which differed in its molecular weight range between isolates (41.7-52.5 kDa for R64, 41.7-53.7 kDa for R38, 39-52.5 kDa for R39, 41.7-53.7 kDa for R40, and 45.7-57.5 kDa for R41). A final single band seen against all isolates except R41 was of 17.4 kDa for R64, R65, and R40 (all biovar 3), 25 kDa for R38, and 24 kDa for R39.

Out of the P. syzygii isolates tested, bands were only seen against R2 and R102 (faintly). While resembling the band pattern seen with the P. solanacearum isolates, the R2 bands differed in MW, having a faint band at 83 kDa followed by the major band at 54 kDa, a cluster at 41.7-45.7 kDa and two single bands at 30 kDa and 23 kDa.

SD1.2, SD2.3, PS1.3 and PS2.3 (faintly) all produced similar patterns against both R1 and R64 (Plate 3.8). No reactivity was obtained with SD2.1.

All mAbs reacting with more than one band were tested against blotted LPS preparations (Plate 3.9).

No bands were seen with SD5.4 (negative control), SD6.2, SD7.3, SD7.4, PS3.1 and PS3.2.

The characteristic LPS "ladder" pattern of banding was seen with SD1.2, SD2.1 (weakly), SD2.3, PS1.3 (weakly), and PS2.3.

Coomassie blue staining of gels loaded with protease digested samples demonstrated the complete removal of protein.

Plates 3.1 and 3.2

The band patterns obtained after immunoblotting analysis of SDS-PAGE separated bacterial proteins with mAbs SD5.4, SD6.1 (plate 3.1) and SD6.3 (Plate 3.2) are presented.

The bacterial species/isolate tested in each track was:

- 1 : P. syzygii isolate R102.
- 2 : P. syzygii isolate R96;
- 3 : P. syzygii isolate R94;
- 4 : P. syzygii isolate R86;
- 5 : P. syzygii isolate R2;
- 6 : P. syzygii isolate R1

Tracks containing the protein molecular weight standards are identified as "S".

The molecular weights corresponding to the standards are indicated to the right of each plate.

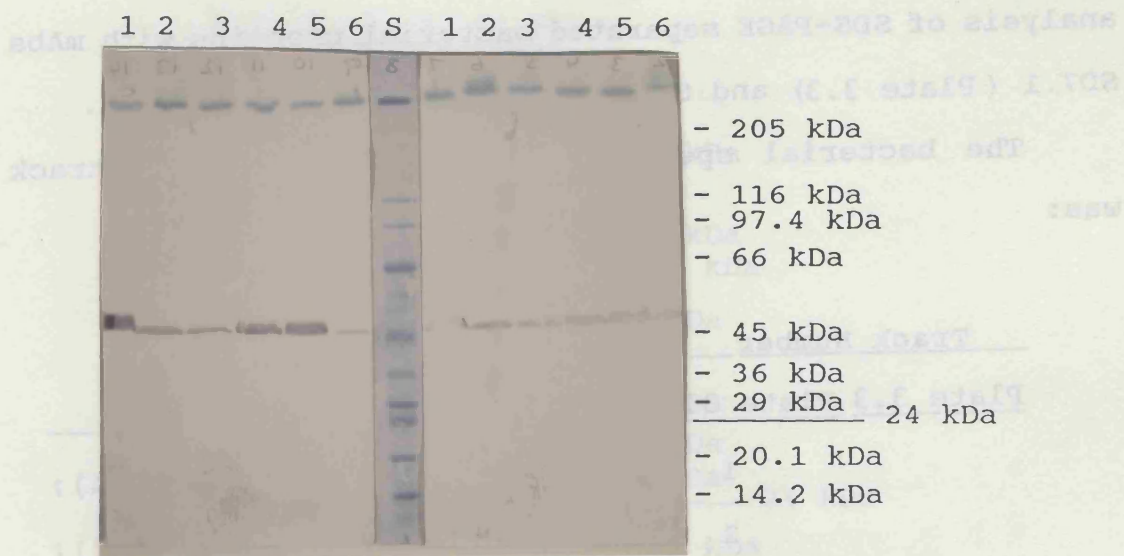


Plate 3.1



Plate 3.2

Plates 3.3 and 3.4

The band patterns obtained after immunoblotting analysis of SDS-PAGE separated bacterial proteins with mAbs SD7.1 (Plate 3.3) and SD7.4 (Plate 3.4) are presented.

The bacterial species/isolate tested in each track was:

<u>Track Number</u>		
<u>Plate 3.3</u>	<u>Plate 3.4</u>	<u>Species/Isolate</u>
	1	<u>P. solanacearum</u> R41 (biovar 4);
	2	" " R40 (" 3);
	3	" " R39 (" 2);
	4	" " R38 (" 1);
	5	" " R65;
	6	" " R64.
1	7	<u>P. syzygii</u> isolate R102;
2	8	<u>P. syzygii</u> isolate R96;
3	9	<u>P. syzygii</u> isolate R94;
4	10	<u>P. syzygii</u> isolate R86;
5	11	<u>P. syzygii</u> isolate R2;
6	12	<u>P. syzygii</u> isolate R1.

Tracks containing the protein molecular weight standards are identified as "S".

The molecular weights corresponding to the standards are indicated to the right of each plate.

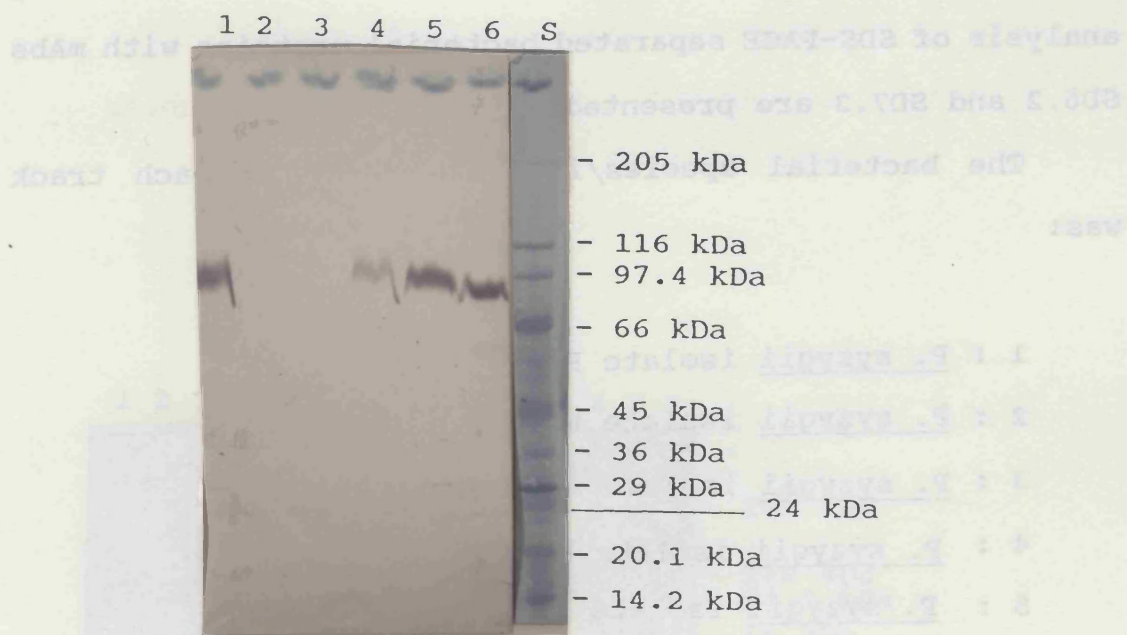


Plate 3.3

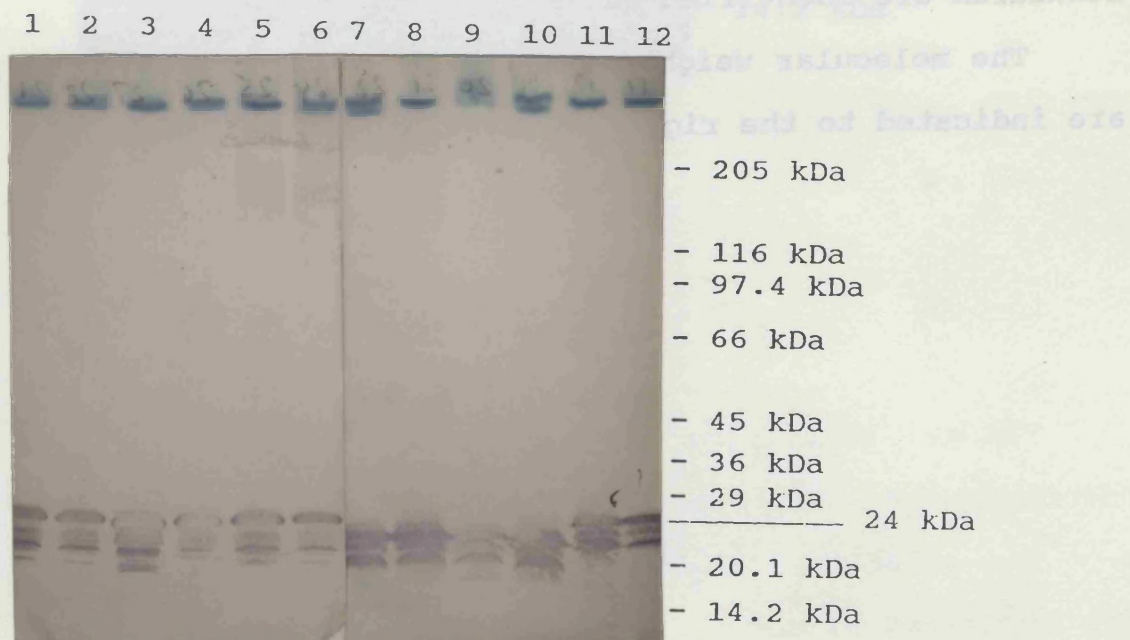


Plate 3.4

Plate 3.5

The band patterns obtained after immunoblotting analysis of SDS-PAGE separated bacterial proteins with mAbs SD6.2 and SD7.3 are presented.

The bacterial species/isolate tested in each track was:

- 1 : P. syzygii isolate R102;
- 2 : P. syzygii isolate R96;
- 3 : P. syzygii isolate R94;
- 4 : P. syzygii isolate R86;
- 5 : P. syzygii isolate R2;
- 6 : P. syzygii isolate R1.

Tracks containing the protein molecular weight standards are identified as "S".

The molecular weights corresponding to the standards are indicated to the right of each plate.

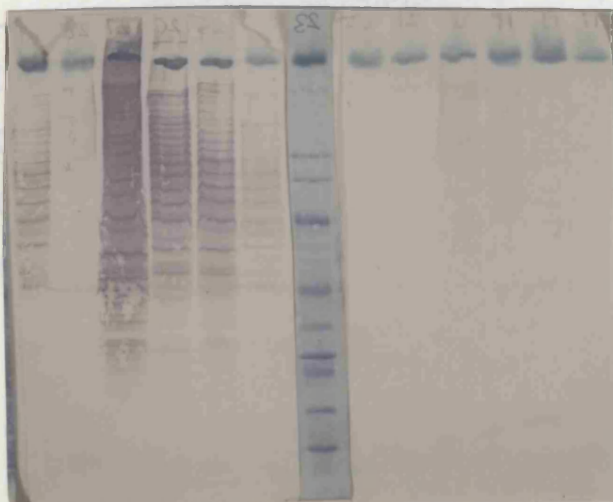
The band patterns obtained after immunoblotting analysis of SDS-PAGE separated bacterial proteins with mAb P3.1 (Plate 3.4) and P3.2 (Plate 3.5) are presented. The bacterial suspensions were loaded in each track

Went:

100 kDa
50 kDa

Track Number

1 2 3 4 5 6 S 1 2 3 4 5 6



- 205 kDa
- 116 kDa
- 97.4 kDa
- 66 kDa
- 45 kDa
- 36 kDa
- 29 kDa
- 24 kDa
- 20.1 kDa
- 14.2 kDa

Plate 3.5

Plates 3.6 and 3.7

The band patterns obtained after immunoblotting analysis of SDS-PAGE separated bacterial proteins with mAbs PS3.1 (Plate 3.6) and PS3.2 (Plate 3.7) are presented.

The bacterial species/isolate tested in each track was:

<u>Track Number</u>		
<u>Plate 3.6</u>	<u>Plate 3.7</u>	<u>Species/Isolate</u>
	1	<u>P. solanacearum</u> R41 (biovar 4);
	2	" " R40 (" 3);
	3	" " R39 (" 2);
	4	" " R38 (" 1);
	5	" " R65;
6	6	" " R64.
1	7	<u>P. syzygii</u> isolate R102;
2	8	<u>P. syzygii</u> isolate R96;
3	9	<u>P. syzygii</u> isolate R94;
4	10	<u>P. syzygii</u> isolate R86;
	11	<u>P. syzygii</u> isolate R2;
5	12	<u>P. syzygii</u> isolate R1.

Tracks containing the protein molecular weight standards are identified as "S".

The molecular weights corresponding to the standards are indicated to the right of each plate.

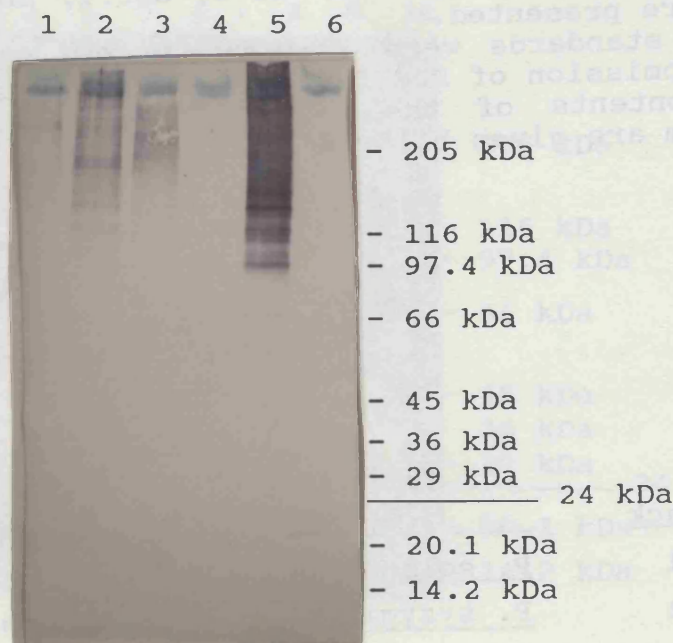


Plate 3.6

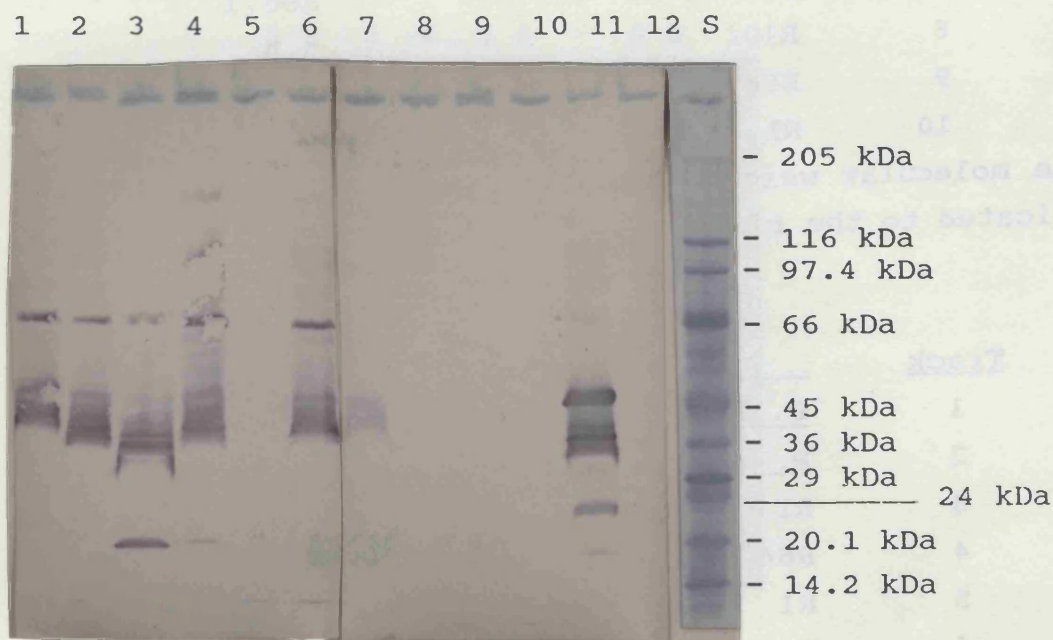


Plate 3.7

Plates 3.8 and 3.9

The results of immunoblotting analysis of SDS-PAGE separated bacterial proteins (Plate 3.8) and LPS preparations (Plate 3.9) with SD1.2, SD2.1, SD2.3, PS1.3, and SD2.3 are presented.

No MW standards were included in the LPS analysis due to the omission of SDS from the gel buffers.

The contents of the tracks, and the mAb tested against them are given below.

<u>Plate 3.8</u>		
<u>Track</u>	<u>Contents</u>	<u>mAb</u>
1	<u>P. solanacearum</u> (R64)	PS1.3
2	<u>P. syzygii</u> isolate R1	"
3	R64	PS2.3
4	R1	"
5	R64	SD1.2
6	R1	"
7	R64	SD2.1
8	R1	"
9	R64	SD2.3
10	R1	"

The molecular weights corresponding to the standards are indicated to the right of each plate.

<u>Plate 3.9</u>		
<u>Track</u>	<u>Contents</u>	<u>mAb</u>
1	<u>P. syzygii</u> isolate R1	SD2.3
2	<u>P. solanacearum</u> (R64)	"
3	R1	SD2.1
4	R64	"
5	R1	SD1.2
6	R64	"
7	R1	PS2.3
8	R64	"
9	R1	PS1.3
10	R64	"

2.2.2. Characterization of Bacterial LPS

LPS preparations of representative *P. syzygii* (R1, R2, R35, R36, R39, R41) and *P. solanaceae* (R54, R55, R56, R57, R58, R59, R60, R61) were compared by SDS-PAGE following the method of Laemmli (1970). The results are shown in Plate 3.8. The major difference between the two species is the presence of two strongly stained bands at 205 kDa and 116 kDa in the *P. syzygii* tracks, which are absent in the *P. solanaceae* tracks. The other bands are similar in both species, with molecular weights of 97.4 kDa, 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa, and 14.2 kDa.

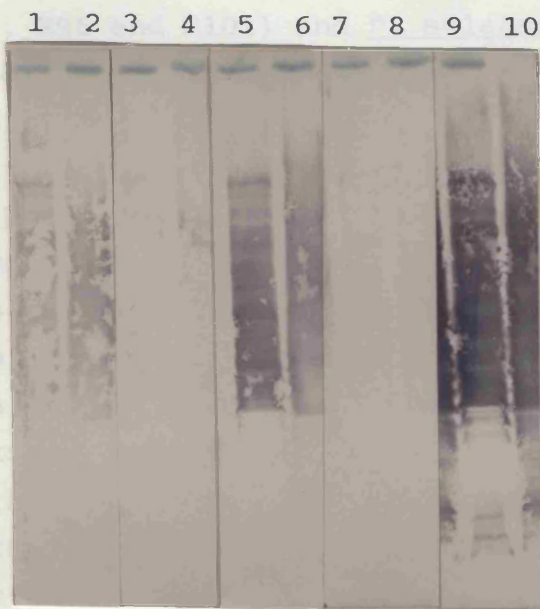


Plate 3.8

The results are shown in Plate 3.9. The major difference between the two species is the presence of two strongly stained bands at 205 kDa and 116 kDa in the *P. syzygii* tracks, which are absent in the *P. solanaceae* tracks. The other bands are similar in both species, with molecular weights of 97.4 kDa, 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20.1 kDa, and 14.2 kDa.

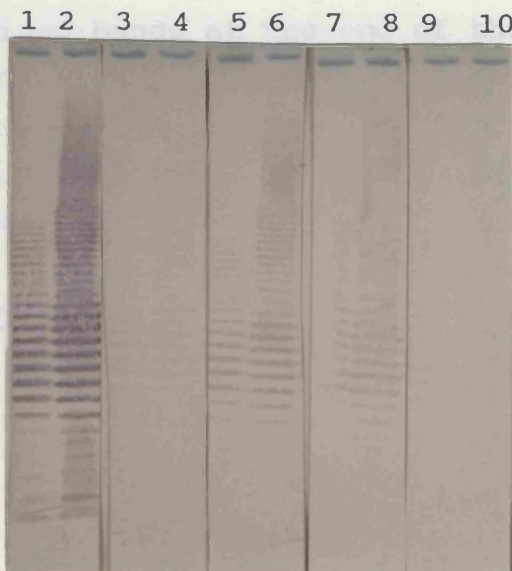


Plate 3.9

3.2.8. Comparison of Bacterial LPS.

LPS preparations of representative P. syzygii (R1, R2, R86, R94, R96 and R102) and P. solanacearum (R64, R65, R38, R39, R40 and R41) isolates were compared by SDS-PAGE followed by silver staining of the gels.

Only minor variations in LPS patterns were evident between P. syzygii isolates (Plate 3.10), although R102 did have some "doublet" bands which were not seen with the other isolates.

Similarly, the LPS patterns of the P. solanacearum isolates did not vary greatly, even between biovars. The only exception was R41, which like R102, did have some doublet bands. The differences in mobilities between some of the tracks was probably due to gel conditions.

The LPS of one P. syzygii isolate (R1) was compared with P. solanacearum (R64, R38, R39, R40 and R41) in adjacent tracks (in order to minimize the effect of intra-gel variation) (Plate 3.11). This gel allows a direct visual comparison, and demonstrates the similarity of the LPS patterns of P. syzygii and P. solanacearum. The major difference between the two species is the presence of two strongly stained bands at the end of the P. syzygii tracks as opposed to one in the P. solanacearum tracks. This difference may be due to sample loading as the faint second band is visible with P. solanacearum and the P. syzygii tracks appear to have received a greater sample loading. The markedly different LPS patterns of P. fluorescens and P. cepacia (both from each other and P. syzygii and P. solanacearum) accentuates the similarity between the P. syzygii and P. solanacearum patterns.

Plates 3.10 and 3.11

Bacterial LPS preparations were separated by SDS-PAGE and the bands patterns visualized by silver staining.

The bacterial species/isolate tested in each track was:

<u>Track Number</u>		
<u>Plate 3.10</u>	<u>Plate 3.11</u>	<u>Species/Isolate</u>
1	1,3,5,7,9,11	<u>P. syzygii</u> isolate R1.
2		<u>P. syzygii</u> isolate R2;
3		<u>P. syzygii</u> isolate R86;
4		<u>P. syzygii</u> isolate R94;
5		<u>P. syzygii</u> isolate R96;
6		<u>P. syzygii</u> isolate R102;
7	2	<u>P. solanacearum</u> R64
8		" " R65
9	4	" " R38 (biovar 1)
10	6	" " R39 (" 2)
11	8	" " R40 (" 3)
12	10	" " R41 (" 4)
	12	<u>P. fluorescens</u> R42
	13	<u>P. cepacia</u> R44

3.1. Discussion

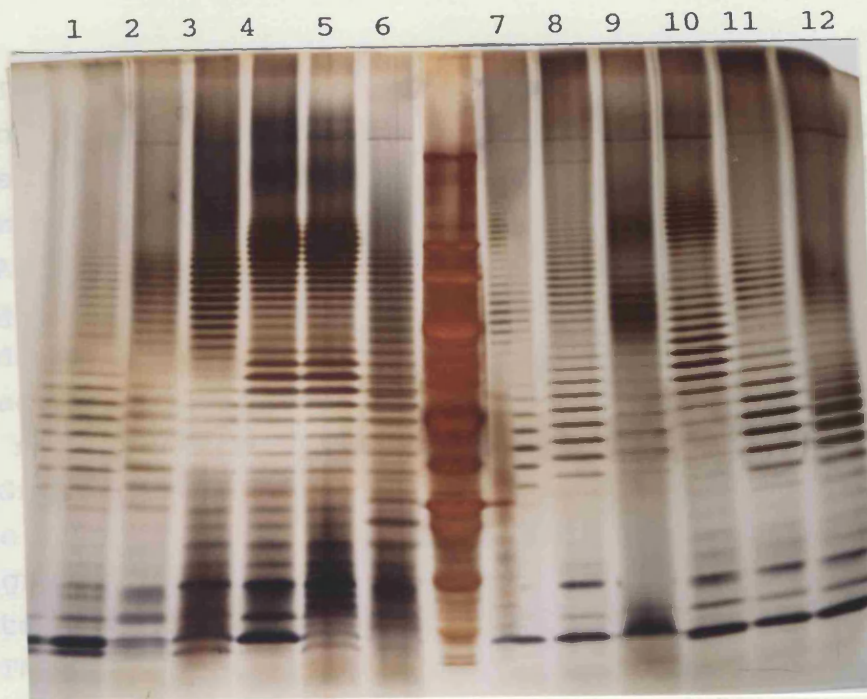


Plate 3.10

1 2 3 4 5 6 7 8 9 10 11 12 13

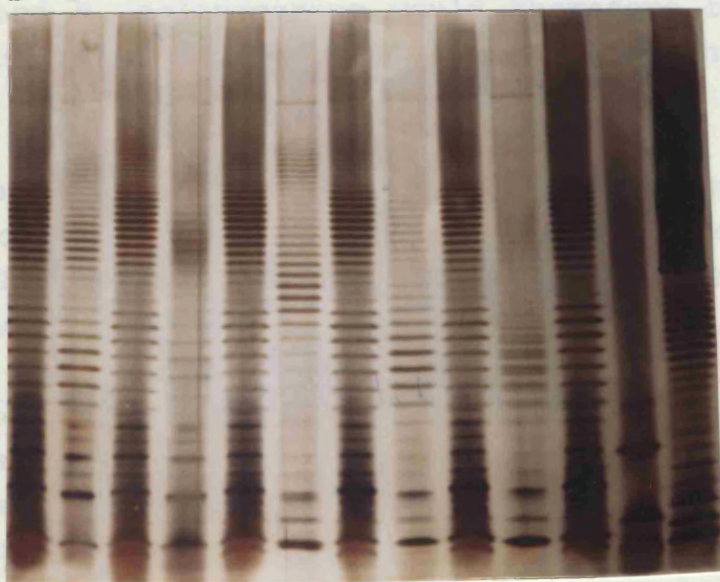


Plate 3.11

3.3. DISCUSSION

The data presented in this chapter both confirm the original reports of a close serological relationship between P. syzygii and P. solanacearum and extend the understanding of that relationship.

Antisera produced in mice to both P. syzygii and P. solanacearum reacted with all isolates of both species included in the mAb testing panel. Both antisera also reacted with isolates of the Banana Blood Disease bacterium which may also be closely related to both species (Dr. S.J. Eden-Green, personal communication). Apart from reactivity of the anti-P. solanacearum antiserum with P. aeruginosa, no significant reactivity was seen with any of the other isolates tested.

This both eliminates the possibility of the cross-reactivity being due to a common bacterial component and also adds further evidence to the close relationship of the two species.

Most of the specific mAbs produced in fusions SD5, SD6, SD7 and PS3 did not react with heat fixed bacteria, suggesting that much of the specificity is located on heat sensitive antigens. The effect of heat fixation on P. syzygii and P. solanacearum would therefore be to reduce the serological differences between them, which may explain why only cross-reactive mAbs were obtained in the rat fusions.

Because the mAbs were selected after screening against fixed whole bacterial cells, it would seem reasonable to assume that only mAbs reactive with surface components would have been selected.

The use of whole cell (rather than OM) preparations in immunoblotting for the characterization of the molecules recognized by the mAbs was based on this assumption.

Three basic band patterns were obtained after immunoblotting analysis of bacteria separated by SDS-PAGE.

MABs SD5.4, SD6.1, SD6.3, and SD7.1 were each reactive with a single protease sensitive (i.e. protein) molecule. While the MW of the protein detected by SD5.4, SD6.1, and

SD6.3 was the same, the ELISA reactivity profiles of these mAbs differ both quantitatively and qualitatively from each other, suggesting that they are different mAbs reactive with the same molecule.

The consistent differences between the intensity and size of the bands from different isolates do not correlate with ELISA values and are unlikely to be due to variations in sample loadings (particularly since the same pattern was not seen with the molecules detected by other mAbs) and may be due to constitutive or inducible variation in the quantitative expression of the protein between different isolates.

SD7.1 reacted with a different (heavier) OM protein. The thickness of the band probably accounts for the variation in mobility seen in the different tracks and suggests that it is present in high numbers on the bacterial cell surface.

MABs SD1.2, SD2.1, SD2.3, PS1.3, and PS2.3, produced similar patterns of multiple bands against a smeared background with whole cell preparations, and were shown to be binding to LPS on blots using protease digested bacteria.

The LPS pattern only became clearly visible on the latter blots as it does not transfer well in the presence of SDS.

MABs SD6.2, SD7.3, SD7.4, PS3.1 and PS3.2 also gave either similar patterns to the LPS reactive mAbs or reacted strongly with several bands. However, none of them showed reactivity with blotted LPS preparations.

The observation that the molecule detected by mAbs SD6.2, SD7.3, and PS3.1 is protease sensitive (i.e. not LPS) and present in a wide range of molecular weights, suggests that it may be a protein Ag that is strongly bound to the peptidoglycan layer. The production of fragments of differing size after cell breakage, all with bound Ag, might be expected to produce the observed band pattern.

Both porin proteins (Lugtenberg & van Alphen, 1983) and the lipoprotein (Braun & Bosch, 1973) that links the OM to the peptidoglycan have been reported to form complexes with peptidoglycan after cell breakage.

The lipoprotein is unlikely to be exposed on the cell surface and would not be accessible for mAb binding in ELISA. In addition, the protein, in order to be both recognised in ELISA (i.e. be at the cell surface) and bound to peptidoglycan, would need to span the OM. This suggests that the Ag recognised by these mAbs is a porin or other protein that spans the OM.

The molecule detected by PS3.2 is a protein as demonstrated by its sensitivity to protease. The pattern of multiple bands obtained suggests that some proteolysis of the protein occurred during cell breakage. This is supported by the presence of the same major band (79 kDa) in all the P. solanacearum tracks (with the exception of R65).

The subsequent pattern of lower MW bands differs between isolates both in the range of MW of the main cluster and also that of the final band. Each of the four biovar isolates displayed a different band pattern, suggesting that PS3.2 is able to differentiate between the biovars of P. solanacearum. The patterns of the three biovar 3 isolates were almost identical, but as only one isolate of biovars 1,2, and 4 were tested, no firm conclusions can be drawn.

The faint band at 177 kDa seen in the R38 and R64 tracks is approximately twice the MW of the main band (76 kDa) and may represent a dimer of the protein. Porin proteins are known to exist as oligomers (probably as trimers) in vivo (Angus & Hancock, 1983; Palva & Randall, 1978; Palva & Westerman, 1979), dissociation of the oligomers occurring upon heating. The protein recognised by PS3.2 may therefore be a porin protein, with the dimer indicating incomplete dissociation of the oligomer. Complexes of three or more proteins, if present, would probably not have entered the gel.

Since intra-specific variation of the protein occurs (within P. solanacearum) it would be logical to assume that the protein detected in some isolates of P. syzygii could be an inter-specific variant of the same protein. The fact that the inter-specific variation is greater than the intra-specific differences suggests that the protein may

act as a marker of the evolutionary distance between and within the two species (especially as reactivity of PS3.2 appears to be almost completely restricted to P. syzygii and P. solanacearum).

The reason for the lack of reactivity of the mAb with the remaining P. syzygii isolates is not clear but may be due to evolutionary loss of the specific determinant (rather than the protein), or to non-expression of the protein under the cultural conditions used.

Purification of the protein using PS3.2 and the production of a panel of mAbs to it could yield interesting information both about the protein and the relationship between the two species. Interestingly, PS3.2 also reacts with one isolate of the blood disease bacterium which is thought to be a member of the P. syzygii group.

SD7.4 produced a similar pattern against all P. solanacearum and P. syzygii isolates and represents a second OM protein shared (again, almost exclusively) between the two species.

The mAbs produced in this study have demonstrated the presence of antigenically related LPS in P. solanacearum and P. syzygii and at least two similar or identical OM proteins (as detected by PS3.2 and SD7.2). This suggests that the serological cross-reactivity is due to evolutionary divergence rather than convergence (on the basis that while convergent evolution may result in a single shared feature, multiple shared features are more likely to stem from a common ancestor), unless it is based on adaptation to a similar habitat. Neither the LPS nor the proteins are found in any of the other bacteria tested except for the BDB (PS3.2 and SD7.4), P. cepacia (SD1.2 (weakly reactive) and SD7.4), and P. fluorescens (SD1.2, SD2.1, and SD2.3 (all weakly reactive)) which are both also closely related to P. solanacearum.

OM proteins are often highly conserved among a given species of bacteria (Hancock et al, 1982; Hofstra & Dankert, 1979; Nicas & Hancock, 1980), and may be species specific or shared by groups of closely related species or genera (Mutharia et al, 1985).

The mAbs described in this chapter have identified at

least three OM proteins which are apparently unique to, and present in most isolates of, P. syzygii. In addition, one OM protein was identified (by SD7.4) that is present only in (all) isolates of P. syzygii and P. solanacearum, and some other closely related bacteria. The presence of these proteins adds further support to the grouping by Roberts et al., (1990) of the "P. syzygii" isolates into a single species (P. syzygii) and to its close relationship to P. solanacearum.

Serotyping of Salmonella and other related genera is based on agglutination reactions with antisera rendered specific by absorption with cross-reacting Ags (Kauffmann, (1966), cited by De Boer et al., (1979)) and is based on differences in the LPS "O" Ag.. Similarly De Boer et al., (1979;1985) were able to subdivide isolates of Erwinia carotovora into serogroups based upon LPS "O" Ag. (De Boer & McNaughton, 1987). Cross-reactions between serogroups were found to be due to flagellar Ags (De Boer, 1980).

The "O" side chains may also contain residues shared by related species (Perry et al., 1986).

O-specific antisera are often produced by denaturation of surface Ags by heat fixation of the bacteria (Edwards & Ewing (1972), cited by De Boer et al. (1979)).

The similarity between the band patterns of P. syzygii and P. solanacearum obtained with the anti-LPS mAbs in immunoblotting suggests the presence of structurally similar LPS in the two species. Silver staining of LPS after SDS-PAGE confirmed the similar band pattern, and accentuates the similarity by comparison with the LPS of two related Pseudomonas species which gave markedly different patterns.

It was not determined as to whether the cross-reacting determinants were present in the "O" side chains or the LPS core. This could have been shown by comparison of identical blotted and silver stained tracks, absence of the fastest running gel band (the core) from the blot would suggest reactivity with the side chains, its detection would suggest that it contained the relevant epitope.

The data obtained about the LPS of the two species can only be used to suggest a relationship at the genus level.

The finding that mAbs SD5.1, SD5.2, SD5.3, and PS3.2 constitute a panel that identifies groups (serogroups) related to the geographical origin of P. syzygii isolates reinforces the findings of Roberts et al. (1989) who differentiated P. syzygii isolates into two groups (I and II) on the basis of cultural and biochemical tests. Fatty acid profiles of a limited number of isolates also produced two distinct groups, corresponding to groups I and II.

Group I only contains Sumatran isolates, and all Javan isolates are contained within group II, although group II also contains some Sumatran isolates. The groups were suggested as representing ecotypes of P. syzygii.

From those isolates in serogroups 1-4, and with the exception of R53, there is an absolute separation of Sumatran (serogroups 1, 2, and 3) and Javan (serogroup 4) isolates, whereas the ecotypes do not give such an absolute distinction due to the inclusion of several Sumatran isolates in group II. In addition, the ecotypes do not extend the distinction to the same level as do the serogroups.

The ecotype groupings however, are more comprehensive than the serogroups since they include most of the isolates that were unclassified (or in group 5) by the mAbs.

The similarity of the results obtained with the serotypes to those of the ecotypes is further evidence of the existence of geographical variants of P. syzygii, particularly since the features upon which the serotypes are based (i.e. surface molecules) are likely to be different from those upon which the ecotype groups are based (i.e. biochemical differences which are likely to be internal, and fatty acid profiles).

Similar findings have been reported by Alvarez et al. (1985), who produced a panel of mAbs that grouped and subgrouped strains of X. campestris pv. campestris. While not as distinct as that reported here, a correlation between the serogroup and geographical origin of isolates was noted.

Ecotypes of P. syringae have been described, based on bacteriophage and bacteriocin typing (Gross et al., 1985).

There was no correlation between ecotype and pathogenicity or other factors.

No correlation is evident between isolates in the different serogroups/ecotypes and their pathogenicity, and it may be that the observed differences reflect either an adaptation to environmental differences between different areas, the existence of different wild strains of P. syzygii, or antigenic changes in isolated populations after initial spread of the organism from a single origin.

The mAbs in the serotyping panel were unfortunately not well characterized since only PS3.2 worked in immunoblotting (and was only available in limited quantities). Neither PS3.2 nor SD5.1 (which was unreactive with heat-fixed bacteria) were reactive with LPS. It is possible that SD5.2 and SD5.3 were reactive with LPS since they reacted with heat fixed bacteria, but this is not sufficient proof as they could be reacting with a heat-stable protein like that detected by SD7.3. The molecular basis for the serotyping of P. syzygii therefore differs from many described systems in not being based purely on LPS differences. It is not a unique situation however, as serotyping based on both OM proteins and LPS has been described (Frasch et al., 1985).

The range of detection limits of the individual mouse mAbs (from 8×10^4 to 1.6×10^6 bacterial cells) reflects the diversity of the target molecules involved. MAbs SD5.4, SD6.1, and SD6.3, which detected identical bands in immunoblotting, had very similar detection limits, further suggesting that they were binding to the same molecule.

The working dilutions used were based on partially purified mAbs, and as such can only relate to the preparations used in this work.

Ideally the mAbs would have been purified further (e.g. by Protein A affinity chromatography) which would have allowed better standardization and comparison of results.

The detection limits are still valid however, as they are based on the use of mAb at a defined point in the titration curve. However, due to the fact that an unknown amount of Ag would have desorbed from the plate during the

ELISA, the detection limits can only be taken as a comparative value (all assays were performed under the same conditions) based on the amount of Ag originally added to the wells.

A mixture of the seven P. syzygii specific mAbs (SD.SM) resulted in a detection limit lower (but not substantially so) than obtained with any of the constituent mAbs. The mixture was used in all subsequent testing since it decreases the chances of false negative results occurring with bacteria deficient in one or more of the molecules detected by the mAbs.

While the mAbs were all used at their individual working dilutions in the mixture, it is likely that the conditions were not optimal, particularly if some mAbs were antagonistic towards others. A more thorough investigation into the interaction of the mAbs with each other, possibly by the use of a chequerboard ELISA, may have resulted in a further lowering of the detection limit.

All of the mAbs in the specific mixture reacted equally well with either acetone fixed or unfixed bacteria thus allowing the option of using either fixed or untreated (i.e. fresh) samples in the assays.

CHAPTER 4

ASSAY DEVELOPMENT

4.1. INTRODUCTION

Screening and characterization of the mAbs required an assay that could handle large number of samples and supply quantitative, comparative data whilst requiring the minimum of development.

A direct Ag binding ELISA (DAB-ELISA) in which bacteria were bound directly to the solid phase fulfilled these criteria. Binding of mAbs could then be detected using an anti-species 2nd Ab conjugate. The only alteration required to standard protocols was the inclusion of dried milk powder suspension as a blocking agent, as preliminary testing revealed a high level of non-specific binding of immuno-reactants to the immobilized bacteria, particularly P. solanacearum (data not presented). The nature of this non-specific binding was not determined, but as it was not prevented by Tween 20 which prevents the formation of hydrophobic interactions, the involvement of other interactions is implied.

Being the assay by which the mAbs were selected and also because of its simplicity and capacity to process large numbers of samples, it was initially intended that the DAB-ELISA would be further developed for diagnostic use with wood and insect samples in Indonesia.

Unfortunately, preliminary work showed the assay to be unsuitable for both applications (data not presented). Wood samples could not be directly applied from saturated clove wood sections to the 96 well microtitre plates. Centrifugal extraction of bacteria into tubes followed by application of the resuspended pellet to plates resulted in the detection of bacteria but was cumbersome, difficult

to standardize and gave low OD values. Direct elution into the wells of 24 well tissue culture plates using KOH gave erratic results possibly due to interference of binding by other substances eluted from the wood. The binding of bacteria in insect homogenates was found to be completely inhibited, again probably by material present in the homogenate.

In addition, it has been found that up to 68% of non-covalently absorbed Ag may be desorbed from the solid phase during an ELISA (Engvall et al. 1971). Relatively large Ags such as bacteria might be expected to be particularly prone to desorption, and some workers have reported total loss of Ag by the end of an assay when whole bacteria were used (Boonekamp, 1989). Repulsion between the negatively charged polystyrene microtitre plate and bacterial cells (which have an overall negative surface charge) may also occur. This may not be too important during mAb screening as long as positive controls are included to ensure that some Ag remains and may actually be advantageous in that mAbs able to give a good signal with low Ag amounts may tend to be selected for. Similarly comparative quantitative data may also still be obtained.

Loss of Ag would be undesirable in a standardized diagnostic assay, particularly for the testing of samples containing low amounts of Ag, and would cause errors and false negative results.

Stabilization of the attachment of both mammalian (Heusser et al. 1981) and bacterial cells (Boonekamp, 1989) to microtitre plates by glutaraldehyde and/or the positively charged homopolymer poly-L-lysine has been reported. No increase in signal could be detected when these reagents are incorporated into the DAB-ELISA (data not presented).

Because of these problems, several alternative assays were adapted and assessed for their applicability to wood and insect samples. These assays (DAS-ELISA, DIA and IFA) were selected for their potentially high sensitivity, ease

of sample application, greater Ag binding stability, and potential to circumvent inhibition of binding by host material. In addition, they are all based upon similar principles and can use Ag of the same form as the DAB-ELISA, therefore increasing the likelihood that the mAbs would function in them.

A simple slide agglutination test for the identification of P. syzygii from culture plates was also developed.

Initial assessment of the assays was performed using cultured bacteria, followed by testing with wood and insect samples.

For the detection of bacteria in samples of wood, assays were assessed mainly by 2 criteria:

i). the strength of the specific signal obtained, particularly with respect to the non-specific background signal, and therefore the confidence with which results can be interpreted;

ii). the simplicity of the assay, especially with respect to sample preparation and application.

Whereas both P. syzygii infected (either from trees showing SD symptoms or from inoculation experiments) and healthy wood samples were available to allow properly controlled experiments to be performed, such clearly defined material was not available for the insect samples. This was due both to the low incidence of insects known to carry P. syzygii even in infected areas, and also the lack of a reliable confirmatory test for the presence of P. syzygii in preserved insects (isolation from fresh insects is possible but is probably unreliable and takes a week or more). Because of this, and also because of the low numbers of bacteria expected to be present in infected insects, the initial development of the assays for use with insects concentrated on three factors:

i). non-specific binding and background signal produced by insect material;

ii). simplicity of sample preparation and application;

iii). masking or inhibition of binding of both Ag (to the solid phase) and Ab (to Ag) by insect material.

All initial investigations used insects collected from disease free areas.

Since it was not possible to produce infected material (either plant or insect) in the UK, all samples used had to be obtained from the cloves project in Indonesia. Much of the information on suitable preservation methods (i.e. those retaining antigenic activity of the bacteria whilst also allowing for their extraction) for samples was gained from experiments on fresh material during a field trip to Indonesia.

4.2. RESULTS

4.2.1. ELISA

A DAS-ELISA using rabbit anti-P. syzygii IgG (provided by Dr. S.J. Eden-Green, purified by Protein A affinity chromatography) at 10 µg/ml (previously determined as the optimum coating concentration) as the trapping Ab and SD.SM as detecting Ab was compared with the DAB-ELISA for the detection of P. syzygii (from 8×10^7 - 8×10^4 bacteria)

The DAS-ELISA had a detection limit of 6.25×10^5 bacteria, compared with a value of 8×10^4 for the direct binding ELISA. The negative control values (i.e. where either Ag, or both Ag and mAb were omitted) for both assays were similar and low (0.007 - 0.017).

The suitability of both the direct Ag binding ELISA and DAS-ELISA for the detection of P. syzygii in the insect vector was assessed. Insect homogenate was tested for the presence of endogenous peroxidase activity, and the effect of homogenate on non-specific binding of immunoreactants and inhibition of binding of P. syzygii (within the range

of numbers likely to be present in the insects) to assay plates was examined.

Preserved insects were individually homogenized in 100 μ l of PBS, distributed into microtitre plates and incubated at 37°C for 2 hours. The three criteria under investigation were tested as follows:

i). non-specific binding: homogenates were tested against specific mAb (SD.SM), non-specific mAb (PS3.1), and 2nd Ab only. The results were compared to those obtained in uncoated wells;

ii). endogenous peroxidase: homogenates were tested against substrate only, results being compared to those obtained in uncoated wells;

iii). binding inhibition: homogenates containing known amounts of cultured P. syzygii cells (5×10^4 , 1×10^5 , or 5×10^5) were tested against specific or non-specific mAb. The results were compared to those obtained with P. syzygii coated in the absence of homogenate.

The results are summarized in Table 4.1.

While some non-specific binding of immunoreactants by insect material was observed, the absorbance values were constant and still low.

No endogenous peroxidase activity was detected in the insect homogenates.

Both assays detected bacteria in the absence of homogenate, giving similar absorbance values, particularly at the lower bacterial dilutions. However, whereas the values obtained with the DAS-ELISA were virtually unchanged by the addition of homogenate, binding was completely inhibited in the direct Ag binding ELISA.

Table 4.1.
The Effects of Insect Homogenate in ELISA

<u>mAb</u>	<u>Bacteria</u>	<u>A₄₅₀</u>			
		<u>DAB-ELISA</u>		<u>DAS-ELISA</u>	
		<u>+Insect</u>	<u>-Insect</u>	<u>+Insect</u>	<u>-Insect</u>
SD.SM	none	0.050	0.017	0.053	0.035
PS3.1	none	0.048	0.013	0.052	0.034
None ^a	none	0.045	0.011	0.050	0.030
None ^b	none	0.010	0.010	0.010	0.011
SD.SM	5 x 10 ⁵	0.048	0.755	0.220	0.251
PS3.1	5 x 10 ⁵	0.046	0.020	0.046	0.032
SD.SM	1 x 10 ⁵	0.052	0.188	0.130	0.140
PS3.1	1 x 10 ⁵	0.046	0.018	0.045	0.030
SD.SM	5 x 10 ⁴	0.045	0.134	0.120	0.121
PS3.1	5 x 10 ⁴	0.050	0.014	0.047	0.033

Key:

- ^a - 2nd Ab only
- ^b - substrate only

The non-specific binding and endogenous peroxidase properties of insect homogenate in ELISA were investigated. The effect of the presence of insect homogenate on the binding of control bacteria (within the range on numbers expected to be found in infected insects) was also investigated and results compared with binding in the absence of insect homogenate.

4.2.2. DIA

Serial dilutions of unfixed and acetone-fixed cells of P. syzygii and P. solanacearum from 2×10^7 - 5×10^3 cells were applied to nitrocellulose using the Bio-Dot apparatus.

Feint colour was seen with high numbers of P. solanacearum (down to 2.5×10^6 cells). The lowest number of P. syzygii cells giving a colour intensity greater than that seen with P. solanacearum was 2×10^5 for unfixed bacteria, and 5×10^4 for fixed bacteria (plate 4.1).

Attempts to apply bacteria from infected wood directly to nitrocellulose by squeezing the wood with pliers to extrude bacterial ooze was not successful due mainly to insufficient bacteria being extracted (squeezing was difficult because of the hardness of the wood), and also discolouration of the membrane by extruded sap.

Successful elution of bacteria from wood was achieved by KOH elution. KOH eluates from both P. syzygii infected, and healthy wood samples, were tested against both specific (SD.SM) and non-specific (PS3.1) mAb as well as second Ab only and substrate only controls. A good colour reaction was obtained with the P. syzygii infected samples when tested with SD.SM, whilst none was seen with the controls (see plate 4.2). Minimal background coloration was observed. The P. syzygii infected wood contained large numbers of bacteria as evidenced by microscopical examination of KOH exudates.

Preliminary experiments using the Bio-Dot apparatus to apply bacteria in insect homogenates to nitrocellulose were unsuccessful due both to blockage and discoloration of the membrane by the homogenate, thus preventing the binding of bacteria and the visualization of substrate deposition. Removal of large debris from the homogenate by low speed centrifugation, and the inclusion of a filter paper sheet above the nitrocellulose did not result in any improvement.

Plates 4.1 and 4.2

The detection limit of SD.SM in DIA was determined against both unfixed and acetone fixed cells.

The results are shown in Plate 4.1.

The samples were as follows:

- Row A - P. syzygii/unfixed;
- Row B - P. syzygii/acetone fixed
- Row C - P. solanacearum/unfixed;
- Row D - P. solanacearum/acetone fixed.

Serial dilutions of bacteria were applied in columns 1 to 12 starting at 2×10^7 .

A duplicate membrane incubated in normal mouse serum instead of mAb produced no visible colour.

Plate 4.2 shows the results obtained after the elution of clove wood with KOH for 15 minutes. Each strip of nitrocellulose was divided into four 12 x 12 mm squares (A to D). P. syzygii infected wood was eluted onto A, healthy wood onto B and C, and D was left blank. four strips were used, and tested as follows:

- 1 - SD.SM + 2nd Ab + substrate;
- 2 - PS3.1 + " + " ;
- 3 - 2nd Ab + substrate;
- 4 - substrate only.

4.1.3. IFA

P. eryngii cells from stock solution fluoresced strongly when probed with 5D.5M (at 2 x 500M dilution) with the 2 x 500M dilution.

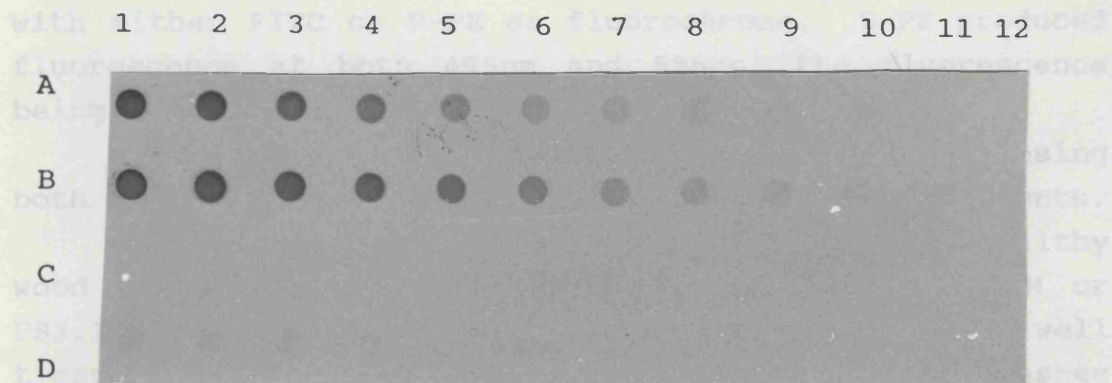


Plate 4.1

Clove wood portions (*P. eryngii* inoculated and healthy) were eluted with KOH onto glass microscope slides. The resulting suspension was spread into a film with a second slide and dried at 40°C followed by fixation in acetone (5 minutes). IFA was performed using the standard protocol except that larger volumes of reagents (approximately 50 µl) were required to cover the smear.



Plate 4.2

4.2.3. IFA

P. syzygii cells from stock solution fluoresced strongly when probed with SD.SM (at 2 x ELISA dilution) with either FITC or R-PE as fluorochrome. R-PE produced fluorescence at both 495nm and 555nm, the fluorescence being stronger at 495nm.

P. syzygii in clove wood was observed by IFA using both thin wood sections and KOH elution of larger segments.

Thin sections of P. syzygii inoculated and healthy wood were incubated in 200 μ l of mAb (either SD.SM or PS3.1) for 15 minutes at 37°C in the wells of 24 well tissue culture plates, followed by five sequential washes in PBS (1 ml) by transfer and immersion in the wells. Following incubation in 2nd Ab (fluorochrome conjugate) and washing, the sections were mounted in phosphate buffered glycerol on glass slides and observed for fluorescence.

Clove wood portions (P. syzygii inoculated and healthy) were eluted with KOH onto glass microscope slides. The resulting suspension was spread into a film with a second slide and dried at 40°C followed by fixation in acetone (5 minutes). IFA was performed using the standard protocol except that larger volumes of reagents (approximately 100 μ l) were required to cover the smear.

Substantial autofluorescence of healthy wood sections at the FITC absorption wavelength (495 nm) prevented the use of FITC on sections (see Plate 4.3).

Only very faint autofluorescence was seen at 555nm, thus allowing the use of R-Pe.

Using R-Pe conjugated 2nd Ab, strong fluorescence of bacterially occluded vessels was observed in P. syzygii inoculated wood sections (see Plate 4.4). Only 30-50% of occluded vessels (as visualized by light microscopy) gave good fluorescence. No fluorescence was seen in the negative control sections.

Fluorescing bacteria were seen in KOH extracts from P. syzygii inoculated wood (Plate 4.5), and not in the negative controls.

Plates 4.3 and 4.4

Thin sections of P. syzygii infected clove wood were incubated sequentially in SD.Sm followed by anti-mouse IgG R-Phycoerythrin conjugate.

The sections were examined at both 495 nm (plate 4.30) and 555 nm (plate 4.4).

Substantial autofluorescence of the section was observed at 495 nm which interfered with the visualization of fluorescing bacteria, whereas specific fluorescence was clearly visible against the background at 555 nm.

Plate 4.5

Plate 6.5 demonstrates the specific detection of P. syzygii by IFA (using FITC) after extraction of bacteria from clove wood with KOH.

Good fluorescence was obtained with both FITC and R-
Fe conjugates. Considerable background fluorescence of

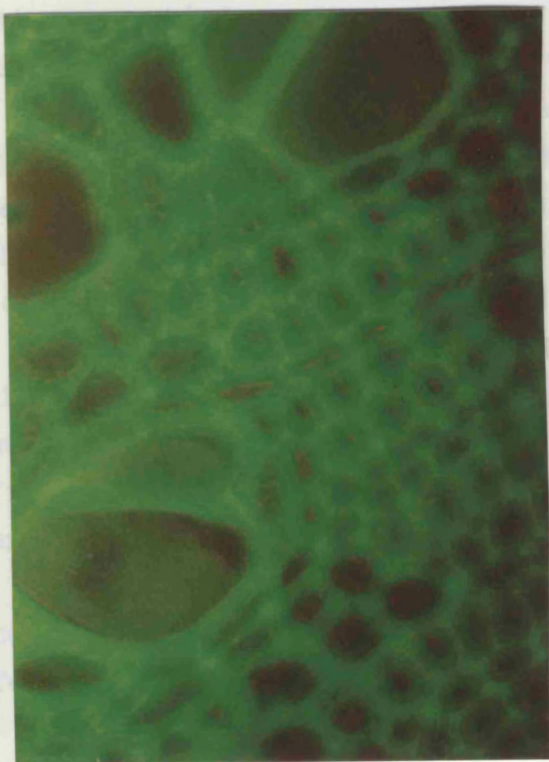


Plate 4.3

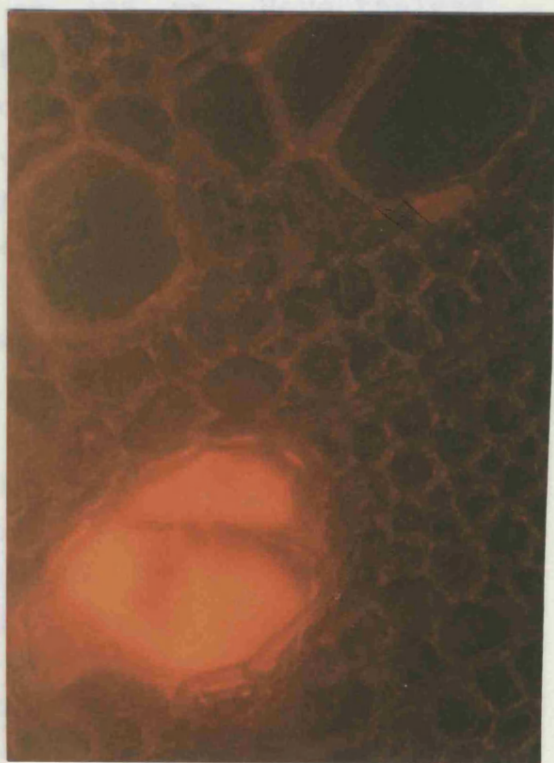


Plate 4.4

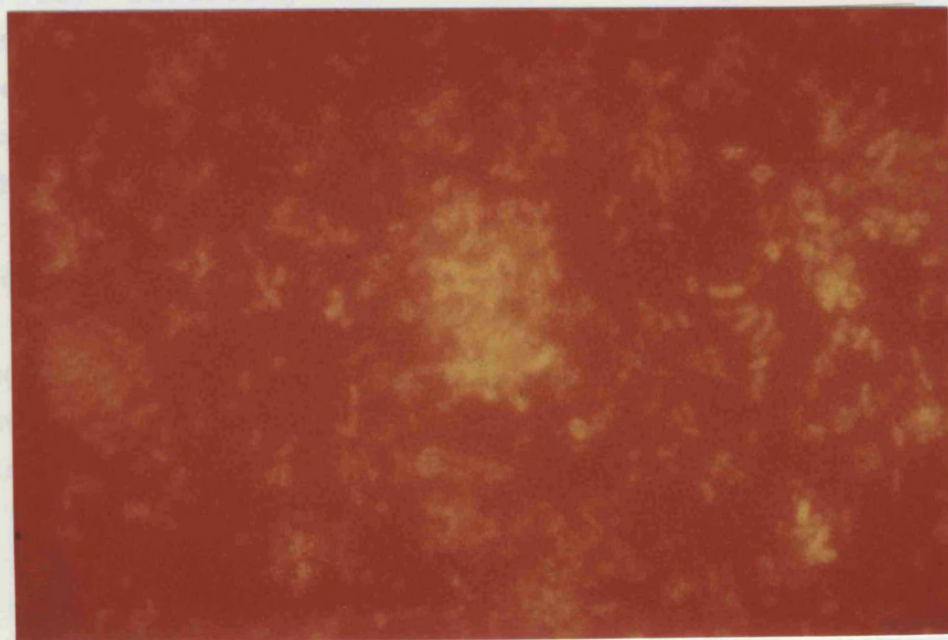


Plate 4.5

Good fluorescence was obtained with both FITC and R-Pe conjugates. Considerable background fluorescence of other particles was observed at 495 nm. Nevertheless, bacteria were identifiable by their morphology.

IFA was assessed for its suitability for detecting P. syzygii in the insect vector. Insect homogenates were tested for non-specific binding and autofluorescence, and the ability of the assay to detect cultured bacteria added to homogenate was assessed.

Single insects were homogenized in 40 μ l of PBS, and the homogenate applied in 10 μ l aliquots to the wells of multiwell microscope slides. After drying (40°C) and fixation in acetone (5 minutes), the IFA was performed as previously described using both FITC and R-Pe conjugates.

Controls included samples incubated in diluent only to test for autofluorescence and non-specific mAb to test for non-specific binding.

Homogenates containing 1×10^5 cultured bacteria per insect were tested against both specific and non-specific mAbs.

Autofluorescence of insect material occurred at 495 nm (FITC) resulting in a uniform background of low intensity fluorescence interspersed with occasional spots of greater intensity. No autofluorescence was observed at 555 nm.

No non-specific binding to either insect material or added bacteria occurred.

Cultured P. syzygii cells added to insect homogenate were clearly distinguishable when tested using the R-Pe conjugate since they appear against a dark background. Brightly fluorescing cells could also be seen, and could be discriminated from the background, when using FITC.

4.2.4. Slide Agglutination

Dilutions of SD.SM from 4x to 1x ELISA dilution were mixed with 50 μ l of P. syzygii or P. solanacearum at 1×10^{10} /ml. mAb at 4x produced agglutination within five minutes, the other dilutions being slower and less complete. No agglutination of P. solanacearum occurred and no autoagglutination of the bacteria in PBS was observed.

4.3. DISCUSSION

The DAB-ELISA might have been expected to be more sensitive than the DAS-ELISA for several reasons:

a). an uncoated plate may present a greater surface area for binding of Ag than a plate coated with trapping Ab, as proteins can only cover approximately 1/3 of the surface due to stearic hindrance (Butler, 1981);

b). the avidity of immobilized Abs for large Ags is reported to decrease by 1-2 orders of magnitude (Parsons, 1981);

c). some Abs may bind to the plastic by their Fab portions and so will not be able to bind Ag (partial denaturation of Ab may solve this as it increases hydrophobicity of F_c thus causing its preferential adsorption (Conradie et al., 1983));

d). bacteria may be too large to be stably bound to an Ab trap with resultant preferential binding of soluble Ags which will present less epitopes for the binding of the detecting Ab;

e). even though purified IgG was used as the trap it would still have contained some non-specific Ab.

The detection limits obtained with the DAB-ELISA using SD.SM were reasonably consistent ($4-8 \times 10^4$ bacteria), whereas those for the DAS-ELISA were more variable. When

originally compared with the DAB-ELISA, the DAS-ELISA had a detection limit of 6×10^5 bacteria, but in the experiment comparing the effect of insect homogenates it was able to detect 5×10^4 bacteria, giving a similar absorbance value to the DAB-ELISA. This variability is unlikely to have been caused entirely by inconsistencies in the preparation of the bacterial dilutions as the DAB-ELISA used the same preparations. The binding of Ag in the DAS-ELISA may be more sensitive to variations in incubation and washing conditions.

The theoretically greater sensitivity of the DAB-ELISA was probably not achieved due to loss of bacteria from the plate during the assay procedure. This loss would appear to be reasonably constant however.

The absorbance values obtained with the DAS-ELISA at the low bacterial numbers likely to be present in the vector would be too low to allow its confident application as a diagnostic test. The results obtained however, did suggest that it would be applicable to the testing of insect samples if it could be made more sensitive. It may have been possible to increase the sensitivity in a number of ways, including:

- i). optimizing the assay conditions to allow for maximal binding and minimal Ag desorption;
- ii). using a trapping Ab of greater avidity or purity;
- iii). increasing the avidity of the mAb mixture (as suggested in chapter 3).

Unfortunately insufficient time was available for this to be done.

The detection limit of SD.SM in DIA (using the Bio-Dot) was similar to that in ELISA. The Bio-Dot allows the application of large volumes of sample to a small area of nitrocellulose and is thus well suited to the testing of dilute samples. The major limitation to this is the amount of interfering substances present in the sample, such as was found with the insect homogenates.

The Bio-Dot was not used to test wood samples as

direct elution onto nitrocellulose by KOH offered a much simpler test method. The results were obtained with wood containing high numbers of bacteria and it is likely that for wood samples containing low numbers of bacteria (e.g. in early or latent infection) the bacteria would be too few, and too widely spread on the membrane for a good signal to be obtained. This could be overcome either by directly eluting several samples from a tree onto the same area of nitrocellulose, or by eluting into an aqueous solution which could then be applied to a much smaller area using the Bio-Dot.

In contrast to the insect homogenates and the direct application of bacterial ooze, no interfering discolouration of the membrane or non-specific reactions occurred with KOH eluted wood samples, thus allowing results to be confidently recorded by eye. Extensive discolouration of the underlying filter paper was observed however, suggesting that the KOH may solubilize wood sap and possibly also prevent it from binding to the membrane. Bacteria would be trapped on the membrane as they are unable to pass through the pores, with binding being stabilized upon subsequent transfer of the membrane to the blocking solution.

The discolouration of the nitrocellulose by insect homogenate prevents the use of DIA for testing insect samples. In addition, while DIA is able to detect bacterial numbers as low as 5×10^4 , the signal obtained at such numbers is weak and would probably be obscured by the background coloration. It may be possible to remove the colour from the homogenate by using an organic solvent such as chloroform, but this would increase the complexity of the assay as well as involving the use of a hazardous chemical.

The DIA should allow simple testing of clove trees for the presence of P. syzygii infection. Samples could either be taken to the laboratory for testing, or eluted onto the membrane in the field for subsequent laboratory testing.

Since the DIA requires no expensive equipment it can be performed under reasonably basic conditions with a minimum of operator training.

IFA was successfully applied to both KOH eluted bacteria and directly to bacteria in clove wood sections.

The fact that whole fluorescing bacterial cells were seen in the IFA of eluted bacteria confirmed that KOH elutes, at least in part, whole cells as opposed to soluble bacterial components.

IFA of thin sections was only possible using R-Pe (at 555 nm) as autofluorescence of the sections (including plugged xylem vessels) at the FITC wavelength made interpretation impossible. The autofluorescence could not be eliminated by the use of filters on the microscope.

Brlansky et al. (1982) reported the successful detection of xylem-limited bacteria in the xylem vessels of thin sections using TRITC. Two TRITC conjugates were tested in this study, but produced either very weak or no fluorescence when tested against cultured P. syzygii preparations (results not shown).

The fluorescence of only a fraction of the plugged xylem vessels could have been due to a number of factors including bacterial degradation resulting in loss of antigenicity, failure of the mAbs to penetrate all vessels, or tylose formation in response to infection. The trapping of soluble Ags secreted by bacteria further down the vessel might result in specific fluorescence of such tyloses. Non-fluorescing tyloses may either be in vessels containing low numbers of bacteria or where the Ag has been trapped by tyloses further down the vessel.

Homogenization of the insects produced a suspension that consisted mainly of single cells, clumps of cells and some larger pieces of tissue, suggesting that it should be sufficient to expose any bacteria present in the insect.

Bacteria could be seen against the background fluorescence at 495 nm but the presence of some intensely fluorescing insect material prevents the use of FITC in a

diagnostic test.

Specifically fluorescing bacteria are easily identified with R-Pe at 555 nm however, since they are seen against an otherwise dark background.

P. syzygii was successfully identified in clove wood by both DIA and IFA. Both assays allow the direct application of bacteria from the sample to the solid phase, and gave clear positive signals with minimal background.

DIA allows the processing of multiple samples with a simpler assay procedure than ELISA and produces clear (and permanent) results with minimum equipment requirements. Because membranes are stable for some time after sample application, DIA would allow samples to be applied in the field for subsequent testing in the laboratory.

The IFA is less applicable to the testing of multiple samples because, while the assay procedure is quick and simple, subsequent microscopic examination can be time consuming and tedious in addition to requiring expensive equipment and a reasonable degree of operator competence.

IFA may be more applicable to the detection of very low numbers of bacteria which is important in testing for early and latent infection. KOH extraction would be the best method to use in such cases as it allows the testing of much larger wood samples than do thin sections and has the additional advantage of working with FITC whereas in vivo testing would require the more expensive R-Pe. By reducing the area of the slide over which the extract is smeared, the number of microscope fields that would have to be examined would be greatly reduced, thus making the search for very low numbers of bacteria more feasible.

The ideal situation in the testing of clove trees would therefore be to first test samples by DIA, and then to test any trees that were negative in DIA by IFA.

The slide agglutination assay, while not very sensitive, may be applicable to the rapid testing of samples containing large numbers of bacteria such as bacterial ooze from heavily infected trees or colonies from

isolation plates.

Neither ELISA nor DIA appear suited for the detection of P. syzygii from insects due to lack of sensitivity, inhibition of binding (DAB-ELISA), low readings (DAS-ELISA), and interference by background colour (DIA).

The DAS-ELISA has the potential for insect testing in that it is able to bind P. syzygii in the presence of insect homogenate, but would need to be made more sensitive.

Only IFA currently presents a potentially suitable method, although further testing on known P. syzygii positive samples is required in order to determine whether bacteria being carried by the insects (as opposed to added cultured bacteria) are exposed to the immunoreactants after sample homogenization.

GENERAL DISCUSSION

The primary goal of the work reported in this thesis was the development of specific, mAb based, immunoassays for the detection of P. syzygii in both clove wood and insect samples.

When the study was begun, very little was known about the physiology, biochemistry, serology and taxonomy of P. syzygii, particularly with reference to its relationship with P. solanacearum. In addition, there was confusion as to the identity of P. syzygii, and as to whether the serological relationship with P. solanacearum was indicative of a close relationship or whether it was due to the presence of shared surface Ags representing common ecological requirements or adaptations.

Work conducted in parallel under the same project revealed the heterogeneity of the P. syzygii isolates, and showed that the relationship with P. solanacearum was not limited to serological cross-reactivity (Roberts et al., 1990). The clarification of the taxonomy of P. syzygii did much to clarify the situation, and to confirm that, while closely related, it was not merely a variant of P. solanacearum.

As well as being the means by which the relationship between P. syzygii and P. solanacearum was first discovered, the potential of serology as a taxonomic tool was further demonstrated in this study.

Antisera, produced in mice to both P. syzygii and P. solanacearum (Table 3.6), were, with very few exceptions, reactive only with isolates from these two species, thus adding further evidence to their close relationship. The antisera also confirmed the findings of Eden-Green et al., (1988) in cross-reacting with the Banana blood disease bacterium, an organism that has only been isolated in Indonesia and which is thought to be another

member of the P. syzygii/P. solanacearum group (Dr. S.J. Eden-Green, personal communication).

Because of the serological similarities between P. syzygii and P. solanacearum, further discrimination was only possible by utilizing the exquisite specificity offered by mAbs. The mAbs produced in this study, in conjunction with SDS-PAGE and immunoblotting, demonstrated the existence of surface molecules unique to both species as well as identifying at least some of those molecules responsible for the cross-reactivity.

A further demonstration of the potential of serology, and in particular mAbs, was the identification of serogroups of P. syzygii. The correlation of the serogroup of an isolate with its geographical origin produced results similar to the P. syzygii ecotype groups based upon cultural and biochemical properties.

The serological variation observed between P. syzygii isolates accentuates the need to screen mAbs against a large panel of isolates to identify mAbs with the desired level of specificity.

This work clearly demonstrates the potential of mAbs as diagnostic reagents, particularly where, as in the case of P. syzygii, polyclonal antisera were not specific enough to allow confident diagnosis.

The time and expense involved in the production of the specific mAbs however, confirms the often cited need (as was the case with P. syzygii) for good scientific and economic justification for such reagents.

The use of a mixture of several mAbs of known specificity and other properties for diagnostic use is clearly advantageous over the use of single mAbs, both in the elimination of problems due to over specificity and also in allowing the detection of a lower number of bacteria, and appears to be so far unique in the field of phytopathogenic bacteriology. Also, whereas some of the individual mAbs may not have functioned in an assay such as IFA (not tested), at least some did, allowing the use

of the mAbs in assays other than the ELISA by which they were selected.

The DAB-ELISA developed in this work provided a useful and efficient tool for the screening and characterization of the mAbs. However, while ELISA is often the method of choice for diagnostic assays because of its simplicity, sample capacity, and sensitivity, it proved to be inapplicable to the detection of P. syzygii in both clove wood and insect samples.

The DIA developed for use with wood samples requires minimal sample preparation and would allow processing of similar numbers of samples to ELISA. In addition, it is simpler to perform than ELISA, allows samples to be applied in the field and tested later, and provides a permanent record of results. DIA is, therefore, more applicable to use in developing countries and the field, than is ELISA. While IFA was also successful in detecting bacteria in wood sections it required the use of the expensive R-phycoerythrin thus making DIA the more economical option.

IFA was the only assay that was able to detect low numbers of bacteria in the presence of insect homogenate, and should be applicable to the testing of insect samples where the necessary equipment and trained staff are available. Although the microscopical examination is time consuming, the assay procedure is simple, and, as with the DIA, samples can be applied to slides remote from the testing facility.

The combination of the specificity of SD.SM and the high sensitivity of the R-phycoerythrin conjugate (the use of which would be justified in this case as it is the only available option), coupled with the absence of autofluorescence of insect material at 555 nm, should produce unequivocal results. In addition, because of its greater sensitivity, IFA would be less likely to produce false negative results than ELISA and DIA.

The use of an anti-species enzyme conjugate as 2nd Ab results in an amplified signal as compared to direct ELISA.

Even with such amplification, the signal obtained in the DAS-ELISA at the low bacterial numbers expected to be present in the insect vector was not sufficiently high enough to allow confident discrimination between low positive, and negative, results.

The avidin-biotin system, involving the use of biotin labelled 2nd Ab and avidin (or streptavidin) labelled enzyme is often claimed to result in enhanced signals, particularly in the case of such reagents as the avidin-biotin-HRP complex (Amersham International). Products from several manufacturers were tested, and, while some resulted in an increase in signal, no improvement in the detection limit of either ELISA or DIA was obtained (results not presented). The increased signal was often accompanied by an increase in the background, and the binding of some avidin reagents to biotin was found to be inhibited by DMP, presumably due to the presence of avidin or an analogue of it in the DMP.

Assay sensitivity may also be increased by the use of fluorescent or luminescent substrates or by enzyme amplification (Bates, 1987). Both approaches have their disadvantages. Fluorescent and luminescent assays both require expensive instrumentation. The only viable enzyme amplification system currently available (from IQ (Bio) Ltd) is expensive and is based on alkaline phosphatase, an enzyme possessed by P. syzygii. Unfixed cells of P. syzygii were observed to hydrolyse p-nitrophenyl phosphate to p-nitrophenol whereas acetone fixed cells did not (data not presented). The assay could therefore be performed by including a fixation step.

If IFA were found to be unable to detect P. syzygii in infected insects, then the expenditure required to develop and perform such assays may be justified.

The minimum number of bacteria detectable by both ELISA and DIA in this work is similar (in order of magnitude) to the values quoted by many other workers (see references quoted in Tables 1.5.2. and 1.5.3). This

suggests that there is a constraint (presumably due to stearic hindrance) to the number of Ab molecules that can be bound to a bacterial cell. Beyond this point, regardless of the number of different Ab specificities present, there would be no increase in binding. If this is the case it reinforces the earlier suggestion for the requirement for a more sensitive detection system for applications with low numbers of bacteria in such assays.

SUGGESTIONS FOR FUTURE WORK

Unfortunately, as is often the case with such projects, time became a limiting factor. The major goals of the project were achieved, in that a specific Ab reagent was produced and incorporated into a number of assay formats for which the potential for use as diagnostic assays was demonstrated. Both the DIA (for wood samples) and the IFA (for insect samples) would benefit from being field tested in Indonesia with a larger variety of samples. The evaluation of the IFA with insect samples that could be tested for the presence of P. syzygii by isolation and culturing of bacteria would be particularly valuable in assessing the accuracy and sensitivity of the assay.

The mAbs could be used to investigate the localization of P. syzygii in the insect vector by immunofluorescent or immunohistochemical testing of sectioned insects with mAb, or to follow the spread of P. syzygii through the vascular tissue during the course of infection.

The molecules determining the serogroups of P. syzygii must presumably correlate with some (as yet unknown) factor (either by their presence or absence) that may be related to environmental conditions in the geographical region from which they were isolated. Identification of the molecules, to the point of determining their function (i.e. involved

in nutrient uptake, adherence, protection from environmental conditions, etc) may be useful in this respect. It may be possible to isolate the molecules by affinity purification from bacterial homogenates using the relevant mAbs in affinity purification, to provide a purified preparation for further analysis.

The serotyping panel is unlikely to be complete (as evidenced by its inability to type all isolates tested) and it may therefore be useful to extend the panel by the production of more mAbs, especially using a wider range of isolates as immunogen.

The P. syzygii isolate panel did not contain isolates from all regions where the bacterium is present. Testing of isolates from these regions might be expected to result in further serogroups and may provide more evidence as to their relevance.

REFERENCES

- Agrios, G.N. (1988). Plant Pathology San Diego. Academic Press.
- Aldwinckle, H.S. & Beer, S.V. (1979). Fire blight and its control. Horticultural Reviews 1, 423-474.
- Allan, E. & Kelman, A. (1977). Immunofluorescent stain procedures for detection and identification of Erwinia carotovora var. atroseptica. Phytopathology 67, 1305-1312.
- Alt, F.W. & Baltimore, D. (1982). Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. Proceedings of the National Academy of Sciences U.S.A. 79, 4118-4122.
- Alt, F.W., Blackwell, T.K., DePinho, R.A., Reth, M.G. & Yancopoulos, G. (1986). Regulation of genome rearrangement events during lymphocyte differentiation. Immunological Reviews 89, 5-30.
- Alvarez, A.M., Benedict, A.A. & Mizumoto, C.Y. (1985). Identification of xanthomonads and grouping strains of Xanthomonas campestris pv. campestris with monoclonal antibodies. Phytopathology 75, 722-728.
- Alvarez, A.M. & Lou, K. (1985). Rapid identification of Xanthomonas campestris pv. campestris by ELISA. Plant Disease 69, 1082-1086.
- Ames, G.F.L., Spudich, E.N. & Nikaido, H. (1974). Protein composition of the outer membrane of Salmonella typhimurium: Effect of lipopolysaccharide mutations. Journal of Bacteriology 117, 406-416.
- Andersson, J. & Melchers, F. (1978). The antibody repertoire of hybrid cell lines obtained by fusion of X63-Ag8 myeloma cells with mitogen-activated B-cell blasts. Current Topics in Microbiology and Immunology 81, 130-139.
- Angus, B.L. & Hancock, R.E.W. (1983). Outer membrane porin proteins F, P, and DI of Pseudomonas aeruginosa and PhoE of Escherichia coli: chemical cross-linking to reveal native oligomers. Journal of

Bacteriology 155, 1042-1051.

- Archer, D.B., Townsend, R. & Markham, P.G. (1982). Detection of Spiroplasma citri in plants and insect hosts by ELISA. Plant Pathology 31, 299-306.
- Armstrong, G.M. & Armstrong, J.K. (1975). Reflections of the wilt Fusaria. Annual Review of Phytopathology 13, 95-103.
- Auger, J.G. & Shalla, T.A. (1974). The use of fluorescent antibodies for detection of Pierce's Disease Bacteria in grapevines and insect vectors. Phytopathology 65, 493-494.
- Autrique, A. & Potts, M.J. (1987). The influence of mixed cropping on the control of potato bacterial wilt (P. solanacearum). Annals of Applied Biology 111, 125-133.
- Barbara, D.J. & Clark, M.F. (1982). A simple indirect ELISA using F(ab')₂ fragments of immunoglobulin. Journal of General Virology 58, 315-322.
- Bates, D.L. (1987). Enzyme amplification in biotechnology. Trends in Biotechnology 5, 204-209.
- Beacham, I.R. (1979). Periplasmic enzymes in gram-negative bacteria. International Journal of Biochemistry 10, 877-883.
- Beckman, C.H. (1987). The Nature of Wilt Diseases of Plants. American Phytopathological Society. St. Paul.
- Bell, J.N., Dixon, R.A., Bailey, J.A., Rowell, P.M. & Lamb C.J. (1984). Differential induction of calcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant pathogen interactions. Proceedings of the National Academy of Science U.S.A. 81, 3384-3388.
- Bennett, C.P.A. Hunt, P. & Ariful Asman, (1985). Association of a xylem-limited bacterium with Sumatra disease of cloves in Indonesia. Plant Pathology 34, 487-494.

- Bennett, C.P.A., Jones, P. & Hunt, P. (1987). Isolation, culture and ultrastructure of a xylem-limited bacterium associated with Sumatra disease of cloves. Plant Pathology 36, 45-52.
- Bennett, R.A. & Billing, E. (1980). Origin of the polysaccharide component of ooze from plants infected with Erwinia amylovora. Journal of General Microbiology 116, 341-349.
- Benz, R. (1988). Structure and function of porins from Gram-negative bacteria. Annual Review of Microbiology 42, 359-393.
- Bernard, O., Hozumi, N. & Tonegaura, S. (1978). Sequences of mouse immunoglobulin light chain genes before and after somatic changes. Cell 15, 1133-1144.
- Billing, E. (1960). An association between capsulation and phage sensitivity in Erwinia amylovora. Nature 186, 819-820.
- Billing, E. (1982). Entry and establishment of pathogenic bacteria in plant tissues. In: Bacteria and Plants The Society for Applied Bacteriology Symposium Series 10, M.E. Rhodes-Roberts & F.A. Skinner (Editors) Academic Press, London. pp 51-70.
- Billing, E. (1987). Bacteria as Plant Pathogens. Aspects of Microbiology 14, Washington. American Society for Microbiology.
- Bollard, E.G. (1960). Transport in the xylem. Annual Review of Plant Physiology 11, 141-166.
- Boonekamp, P.M. (Editor) (1989). Monoclonal antibodies and immunological techniques to detect plant pathogens. Proceedings of the COST-88 Workshop, Wageningen, The Netherlands.
- Bos, E.S., van der Doelen, A.A., van Rooy, N. & Schuurs, A.H.W.M. (1981). 3,3',5,5'-tetramethylbenzidine as an Ames test negative chromogen for horseradish peroxidase in enzyme immunoassay. Journal of Immunoassay 2, 187-204.

- Boucher, C., Barberis, P., Trigalet, A. Ph. & Demery, D. (1985). Transposon mutagenesis of Pseudomonas solanacearum: isolation of Tn5 induced avirulent mutants. Journal of General Microbiology 131, 2449-2457.
- Boucher, C.A., Martinel, A., Barberis, P., Alloing, G. & Zischek, C. (1986). Virulence genes are carried by a megaplasmid of the plant pathogen P. solanacearum. Molecular and General Genetics 205, 270-275.
- Boucher, C.A., Van Gijsegem, F., Barberis, P., Arlat, M. & Zischek, C. (1987). P. solanacearum genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. Journal of Bacteriology 169, 5626-5632.
- Bove, J.M. (1984). Wall-less prokaryotes of plants. Annual Review of Phytopathology. 22, 361-396.
- Bradbury, J.F. (1967). Erwinia stewartii. In: CMI Descriptions of Pathogenic Fungi and Bacteria. Commonwealth Mycological Institute, Kew. 123.
- Bradbury, J.F. (1973a). Pseudomonas caryophylli In: CMI Descriptions of Pathogenic Fungi and Bacteria. Commonwealth Mycological Institute, Kew. 373.
- Bradbury, J.F. (1973b). Xanthomonas vascularum In: CMI Descriptions of Pathogenic Fungi and Bacteria. Commonwealth Mycological Institute, Kew. 380.
- Bradbury, J.F. (1986). Guide to plant pathogenic bacteria. CAB International Mycological Institute London. 332pp.
- Bragg, P.D. & Hou, C. (1972). Organization of proteins in the native and reformed outer membranes of Escherichia coli. Biochimica et Biophysica Acta 274, 478-488.
- Braun, V. (1973). Molecular organization of the rigid layer and the cell wall of Escherichia coli. Journal of Infectious Diseases 128, S9-S16.

- Braun, V. & Bosch, V. (1973). Distribution of murein lipoprotein between the cytoplasmic and outer membranes of Escherichia coli. FEBS Letters 34, 307-310.
- Brlansky, R.H., Lee, R.F., Timmer, L.W., Purcifull, D.E. & Raju, B.C. (1982). Immunofluorescent detection of xylem-limited bacteria in situ. Phytopathology 72, 1444-1448.
- Brlansky, R.H., Timmer, L.W., French, W.J. & McCoy, R.E. (1983). Colonization of the sharpshooter vectors, Oncometopia nigricans and Homalodisca coagulata, by xylem-limited bacteria. Phytopathology 73, 530-535.
- Brown, G., & Ling, N.R. (1988). Murine monoclonal antibodies. In: Antibodies - A Practical Approach (Volume 1) D. Catty (Ed). IRL Press. Oxford.
- Buddenhagen, I.W. (1985). Bacterial wilt revisited. In: Bacterial Wilt Disease in Asia and the South Pacific G.J. Persley (Editor). ACIAR Proceedings No. 13. pp 126-143.
- Buddenhagen, I.W. & Elsasser, T.A. (1962). An insect-spread bacterial wilt epiphytotic of bluggoe banana. Nature 194, 164-165.
- Buddenhagen, I.W. & Kelman, A. (1964). Biological and physiological aspects of bacteria wilt caused by Pseudomonas solanacearum. Annual Review of Phytopathology 2, 203-230.
- Burnet, F.M. (1957). A modification of Jerne's theory of antibody production using the concept of clonal selection. Australian Journal of Science 20, 67-69.
- Burton, D.R. (1985). Immunoglobulin G sites. Molecular Immunology 22, 161-206.
- Butler, (1981). The amplified ELISA: Principles of and application for the comparative quantitation of class and subclass antibodies and the distribution of antibodies and antigens in biochemical separates. Methods in Enzymology 73, 482-523.

- Calzolari, A., Mazzucchi, U. & Gasperini, C. (1982). Cross-reactions between Erwinia amylovora and other bacteria in immunofluorescence staining using different antisera. Phytopath. medit. 21, 110-112.
- Campbell, A.M. (1984). Monoclonal antibody technology. In: Laboratory Techniques in Biochemistry and Molecular Biology (Volume 13). R.H. Burdon & P.H. van Knippenberg (Editors). Elsevier, Amsterdam. 266pp.
- Catty, D. & Raykundalia, C. (1988). Gel immunodiffusion, immunoelectrophoresis, and immunostaining methods. Chapter 6 in: Antibodies: A Practical Approach Vol.1. D. Catty (Ed). Oxford. Irl Press. pp137-168.
- Chatterjee, A.K. & Vidaver, A.K. (1986). In: Genetics of Pathogenicity Factors: Application to Phytopathogenic Bacteria - Advances in Plant Pathology Vol.4. D.S. Ingram & P.H. Williams (Editors) Academic Press, London.
- Chaudhary, V.K., Queen, C., Junghans, R.P., Waldmann, T.A., Fitzgerald, D.J. & Pastan, I. (1989). A recombinant immunotoxin consisting of two Ab variable domains fused to Pseudomonas exotoxin. Nature 339, 394-396.
- Challacombe, S.J. (1988). Application of ELISA to microbiology. In: Elisa and Other Solid Phase Assays D.M. Kemeny & S.J. Challacombe (Eds.). John Wiley & Sons. Chichester. pp 319-342.
- Clark, B.R. & Engvall, E. (1980). in: Enzyme-Immunoassay p167. E.T. Maggio (Editor). C.R.C. Press, Boca, Raton, Florida.
- Clark, M.F., Barbara, D.J. & Davies, D.L. (1983). Production and characteristics of antisera to Spiroplasma citri and clover phyllody-associated antigens derived from plants. Annals of Applied Biology 103, 251-259.
- Clark, M.F., Lister, R.M. & Bar-Joseph, M. (1986). ELISA Techniques. Methods in Enzymology 118, 742-766.

- Cole, M.F., Hsu, S.D. & Bowen, W.H. (1982). A fluorescein linked immunosorbent assay for detection of antibacterial antibodies in secretions and serum. Journal of Immunological Methods 53, 335-343.
- Conradie, J.D., Govender, M. & Visser, L. (1983). ELISA solid phase: partial denaturation of coating Ab yields a more efficient solid phase. Journal of Immunological Methods 59, 289-299.
- Conway de Macario, E., Macario, A.J.L. & Jovell, R.J. (1986). Slide immunoenzymatic assay (SIA) in hybridoma technology. Methods in Enzymology 121, 509-525.
- Cook, D.R. & Sequeira, L. (1988). The use of restriction fragment length polymorphism (RFLP) in taxonomy and diagnosis. Bacterial Wilt Newsletter 4, 4.
- Cook, W.R., MacAlister, T.J. & Rothfield, L.I. (1986). Compartmentalization of the periplasmic space at division sites in Gram negative bacteria. Journal of Bacteriology 168, 1430-1438.
- Coombs, R.R.A., Scott, M.L. & Cranage M.P. (1987). Assays using red cell-labelled antibodies. Journal of Immunological Methods 101, 1-14.
- Cornett, W.C., Vincent, J.W., Gray, W.A. & Falkler, Jr. W.A. (1985). Specificity of monoclonal antibodies reactive with Fusobacterium nucleatum: effect of formalin fixation. Journal of Immunological Methods 84, 321-326.
- Cory, S., Tyler, B.M. & Adams, J.M. (1981). Sets of immunoglobulin V_K genes homologous to ten cloned V_K sequences: Implications for the number of germline V_K genes. Journal of Molecular and Applied Genetics 1, 103-116.
- Costerton, J.W., Irvin, R.T. & Cheng, K.J. (1981). The bacterial glycocalyx in nature and disease. Annual Review of Microbiology 35, 299-324.
- Cotton, R.G.H., & Milstein, C. (1973). Fusion of two immunoglobulin-producing myeloma cells. Nature 244,

42-43.

- Crosse, J.E. (1966). Epidemiological relations of the Pseudomonad pathogens of deciduous fruit trees. Annual Review Phytopathology 4, 291-310.
- Crosse, J.E., Goodman, R.N. & Shaffer, W.H. (1972). Leaf damage as a predisposing factor in the infection of apple shoots by E. amylovora. Phytopathology 62, 176-182.
- Crowley, C.F. & De Boer, S.H. (1982). Non-pathogenic bacteria associated with potato stems cross-react with Corynebacterium sepedonicum antisera in immunofluorescence. American Potato Journal 59, 1-8.
- Dabek, A.J., Jones, P. & Martin, P.J. (1985). Mycoplasma-like organisms in clove trees affected by sudden death disease in Zanzibar and Pemba. Phytopathology Zeitschrift 114, 180-184.
- Daft, G.C. & Leben, C. (1972). Bacterial blight of soybean: epidemiology of blight outbreaks. Phytopathology 62, 57-62.
- Daniel, J.F. & Boher, B. (1981). Fluorescent antibody technique for detection of Xanthomonas campestris pv. manihotis on cassava leaves. Proceedings of the 5th International Conference, on Plant Pathogenic Bacteria, California, U.S.A.
- Darvill, A.G. & Albersheim, P. (1984). Phytoalexins and their elicitors - defence against microbial infection in plants. Annual Review of Plant Physiology 35, 243-275.
- Davis, B.D. (1980). Bacterial nutrition and growth. In: Microbiology B.D. Davis (Editor). Harper & Row Maryland. 1355pp.
- Davis, K.R., Lyon, D.G., Darvill, A.G. & Albersheim, P. (1984). Host - pathogen interaction. XXV. Endopolygalacturonic acid lyase from Erwinia carotovora elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant

Physiology 74, 52-60.

- Davis, M.J., Purcell, A.H. & Thomson, S.V. (1978). Pierce's disease of grapevines: isolation of causal bacterium. Science 199, 75-77.
- Davis, M.J., Whitcomb, R.F. & Graves Gillespie Jr., A. (1981) The Prokaryotes - A handbook on habits, isolation and identification of bacteria. In: Fastidious Bacteria of Plant Vascular Tissue and Invertebrates. Berlin Springer Verlag pp2172-2188.
- Davis, M.J., Gillaspie, A.G., Vidaver, A.K. & Harris, R.W. (1984). Clavibacter: A new genus containing some phytopathogenic coryneform bacteria, including Clavibacter xyli subsp. cynodontis subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermuda grass stunting disease. International Journal of Systemic Bacteriology 34, 107-117.
- Davis, M.J. & Harrison, N.A. (1989). Serological techniques for diagnosis of ratoon stunting disease. In Press.
- De Boer, S.H. (1980). Serological relationships among flagella of Erwinia carotovora var. atroseptica and some E. carotovora var. carotovora serogroups. Canadian Journal of Microbiology 26, 567-571.
- De Boer, S.H. (1982). Cross-reaction of Corynebacterium sepedonicum antisera with C. insidiosum, C. michiganense, and an unidentified coryneform bacterium. Phytopathology 72, 1474-1478.
- De Boer, S.H. (1983). Evaluation of an agar immunodiffusion procedure for confirming bacterial ring rot diagnoses. American Potato Journal 60, 661-669.
- De Boer, S.H., Bradshaw-Rouse, J.J., Sequeira, L. & McNaughton, M.E. (1985). Sugar composition and serological specificity of Erwinia carotovora lipopolysaccharides. Canadian Journal of Microbiology 31, 583-586.

- De Boer, S.H. & Copeman, R.J. (1980). Bacterial ring rot testing with the indirect fluorescent antibody staining procedure. American Potato Journal 57, 457-465.
- De Boer, S.H., Copeman, R.J. & Vrugink, H. (1979). Serogroups of Erwinia carotovora potato strains determined with diffusible somatic antigens. Phytopathology 69, 316-319.
- De Boer, S.H. & McNaughton, M.E. (1987). Monoclonal antibodies to the lipopolysaccharide of Erwinia carotovora subsp. atroseptica serogroup I. Phytopathology 77, 828-832.
- De Boer, S.H. & Slack, S.A. (1984). Current status and prospects for detecting and controlling bacterial ring rot of potatoes in North America. Plant Disease 68, 841-844.
- De Boer, S.H. & Wieczorek, A. (1984). Production of monoclonal antibodies to Corynebacterium sepedonicum. Phytopathology 74, 1431-1434.
- De Boer, S.H. (1987). Use of monoclonal antibodies to identify and detect plant pathogenic bacteria. Canadian Journal of Plant Pathology 9, 182-187.
- De Boer, S.H., Wieczorek, A. & Kummer, A. (1988). An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. Plant Disease 72, 874-878.
- Deisenhofer, J., Colman, P.M., Epp, O., & Huber, R. (1976). Crystallographic structural studies of a human Fc fragment. II. A complete model based on a Fourier map at 3.5 Å resolution. Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie 357, 1421-1433.
- Deverill, I. & Reeves, W.G. (1980). Light scattering and absorption - developments in immunology. Journal of Immunological Methods 38, 191-204.
- Devi, L.R. & Menon, M.R. (1980). Transmission of P. solanacearum through tomato seeds. Agricultural

Research Journal, Kerala 18, 120-122.

- Dietzgen, R.G. & Sander, E. (1984). Monoclonal antibodies for determination of the structure dependent function of the C-terminal region of tobacco mosaic virus (TMV) coat protein (TMV-P). In: Hybridoma Technology in Agricultural and Veterinary Research N. Stern & H.R. Gamble (Editors). Rowman & Allanheld, New Jersey.
- Dimond, A.E. (1962). Wilt pathogens and oxygen levels in tracheal fluid of stems. Phytopathology 52, 7 (Abstr.).
- Dimond, A.E. (1970). Biophysics and Biochemistry of the Vascular Wilt Syndrome. Annual Review of Phytopathology 8, 301-322.
- Doetsch, R.N. & Sjoblad, R.D. (1980). Flagellar structure and function in eubacteria. Annual Review of Microbiology 34, 69-108.
- Domen, H.Y. & Alvarez, A.M. (1978). Detection of Xanthomonas campestris in soil using a direct immunofluorescent technique. In: Proceedings of the 4th International Conference on Plant pathogenic Bacteria, Angers. pp301-305.
- Domin, B.A., Serabjit-Singh, C.J. & Philpot, R.M. (1984). Quantitation of rabbit cytochrome P-450, form 2, in microsomal preparations bound directly to nitrocellulose paper using a modified peroxidase immunostaining procedure. Analytical Biochemistry 136, 390-396.
- Dresser, D.W. (1986). Immunization of experimental animals. In: Handbook of Experimental Immunology (Chapter 8). D.M. Weir (Editor).
- Dristig, M.C.G. & Dianese, J.C. (1988). Diagnostic patterns of cell envelopes. Bacterial Wilt Newsletter 4, p6 (Abstr.)
- Dundas, I.E.D. (1977). Physiology of Halobacteriaceae. Advances in Microbial Physiology. 15, 85-120.

- Duvick, J.P. & Sequeira, L. (1984). Interaction of P. solanacearum, LPS and extracellular polysaccharide with an agglutinin from potato tubers. Applied and Environmental Microbiology 48, 192-198.
- Edelman, G.M. & Gall, W.E. (1969). The antibody problem. Annual Review of Biochemistry 38, 415-466.
- Early, P., Huang, H., Davis, M., Calame, K. & Hood, L. (1980). An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: V_H, D and J_H. Cell 19, 981-992.
- Eden-Green, S.J. & Billing, E. (1974). Fireblight. Review of Plant Pathology 53, 353-365.
- Eden-Green, S.J. (1988). Technical Report for 1982 - 1988 of the Clove Diseases Research Project (Colombo Plan ATA-71). Overseas Development Administration.
- Eden-Green, S.J., Balfas, R., Sutardjo, T., Susniahti, N. & Hasnam, N. (1987a). Preliminary results on transmission of xylem-limited bacteria causing Sumatra disease of cloves in Indonesia by tube-building cercopoids, Hindola spp. (Homoptera: Machaerotidae) In: Proceedings of the 6th International Conference on Plant Pathogenic Bacteria. Maryland, U.S.A., 1985. E.L. Civerolo, A. Collmer, R.E. Davis & A.G. Gillespie (Editors). Martinus Nijhoff Publishers, Dordrecht. pp370-374.
- Eden-Green, S.J., Balfas, R. & Jamalius, (1987b). Transmission of xylem-limited bacteria causing Sumatra disease of cloves by tube-building cercopoids, Hindola spp. (Homoptera:Machaerotidae). In: Proceedings of the 2nd International Conference on leafhoppers and planthoppers of Economic Importance. Provo, Utah, U.S.A., 1986. L.R. Nault & M.R. Wilson (Editors). Commonwealth Institute of Entomology, London. pp101-107.
- Eden-Green, S.J. & Machmud, M. (1988). Letter to the editor. Bacterial Wilt Newsletter. 3, 5.

- Eden-Green, S.J., Supriadi, Hasnam, N. & Hunt, P. (1987c). Serological relationship between the xylem-limited bacterium causing Sumatra disease of cloves in Indonesia and Pseudomonas solanacearum. In: Proceedings of the 6th International Conference on Plant Pathogenic Bacteria. Maryland, U.S.A., 1985. E.L. Civerolo, A. Collmer, R.E. Davis & A.G. Gillespie (Eds). Martinus Nijhoff Publishers, Dordrecht. pp357-361.
- Eden-Green, S.J., Supriadi, & Sri Yuni Hartati. (1988). Characteristics of Pseudomonas celebensis, the cause of blood disease of bananas in Indonesia. In: Proceedings of the 5th International Congress of Plant Pathology. Kyoto, Japan, 1988. p389.
- Edwards, P.R. & Ewing, W.H. (1972). Identification of Enterobacteriaceae. Third Edition. Burgess Publishing Co. MN. 362pp.
- Ehrlich, P.H., Moyle, W.R., Zeinab, M.A. & Canfield, R.E. (1982). Mixing two monoclonal antibodies yields enhanced affinity for antigen. Journal of Immunology 128, 2709-2713.
- Elrod, R.P. (1941). Serological studies of the Erwinieae. I. Erwinia amylovora. Botanical Gazette 103, 123-131.
- Elrod, R.P. & Braun, A.C. (1947a). Serological studies of the genus Xanthomonas. I. Cross-agglutination relationships. Journal of Bacteriology 53, 509-518.
- Elrod, R.P. & Braun, A.C. (1947b). Serological studies of the genus Xanthomonas. II. Xanthomonas translucens groups. Journal of Bacteriology 53, 519-524.
- Elwell, L.P. & Shipley, P.L. (1980). Plasmid-mediated factors associated with virulence of bacteria to animals. Annual Review of Microbiology 34, 465-496.
- Engvall, E., Jonsson, K., & Perlmann, P. (1971). Enzyme-linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody coated tubes.

- Biochimica et Biophysica Acta. 251, 427-434.
- Engvall, E. & Perlmann, P. (1971), Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.
- Esau, K. (1965). Plant Anatomy New York, London, Sydney. John Wiley & Sons.
- Fahy, P.C. & Persley, G.J. (Editors). (1983). in: Plant Bacterial Diseases: A Diagnostic Guide. Academic Press, Sydney. 393pp.
- Fazekas de St. Groth, S. & Scheidegger, D. (1980). Production of monoclonal antibodies: strategy and tactics. Journal of Immunological Methods 35, 1-21.
- Feliciano, A. & Daines, R.H. (1970). Factors influencing ingress of Xanthomonas pruni through peach leaf scars and subsequent development of spring cankers, Phytopathology 60, 1720-1726.
- Fett, W.F. & Jones, S.B. (1983). Interaction of soybean with Xanthomonas campestris pv glycines - an ultrastructural study. Phytopathology 73, 365 (Abstr.).
- Fletcher, J. & Slack, S.A. (1986), Latex agglutination as a rapid detection assay for Spiroplasma citri. Plant Disease 70, 754-756.
- Frasch, C.E., Zollinger, W.D. & Poolman, J.T. (1985). Serotype antigens of Neisseria meningitidis and a proposed scheme for designation of serotypes. Reviews of Infectious Diseases 7, 504-510.
- Freebairn, H.T. & Buddenhagen, I.W. (1964). Ethylene production by P. solanacearum. Nature (London) 202, 313-314.
- French, D.L., Laskov, R. & Scharff, M.D. (1989). The role of somatic hypermutation in the generation of antibody diversity. Science 241, 1152-1157.
- French, E.R. & Sequeira, L. (1970). Strains of P. solanacearum from Central and South America, a comparative study. Phytopathology 60, 506-512.

- Fuerst, J.A. & Hayward, A.C. (1969). Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. Journal of General Microbiology 58, 227-238.
- Galfré, G., Butcher, G.W., Howard, J.C., Wilde, C.D. & Milstein, C. (1980). Clonal competition and stability of hybrid myeloma of mouse and rat origin. Transplantation Proceedings 12, 371-375.
- Galfré, G. & Milstein, C. (1981). Preparation of monoclonal antibodies: Strategies and procedures. Methods in Enzymology 73, 3-46.
- Gampi, L. & Sequeira, L. (1980). Multiplication of *P. solanacearum* in resistant potato plants and the establishment of latent infections. American Potato Journal 57, 319-329.
- Gampi, L., Sequeira, L. & French, E.R. (1980). Latent infection of potato tubers by *P. solanacearum*. American Potato Journal 57, 377-386.
- Garland, P.B. (1977). Energy transduction and transmission in microbial systems. Society for General Microbiology Symposium 27, 1-21.
- Garnier, M., Danel, N. & Bove, J.M. (1984). Etiology of citrus greening disease. Annals Institut Pasteur/Microbiologie 135A, 169-179.
- Garnier, M., Martin-Gros, G. & Bové J.M. (1987). Monoclonal antibodies against the bacterial-like organism associated with citrus greening disease. Annals Institut Pasteur/Microbiologie 138, 639-650.
- Gerisch, G., Luderitz, O. & Roschmann, E. (1967). Antikörper für die Phagozytose von Bakterien durch Amöben Z. Naturforsch 116, 109.
- Glauert, A.M. & Thornley, M.J. (1969). The topography of the bacterial cell wall. Annual Review of Microbiology 23, 159-198.
- Goding, J.W. (1980). Antibody production by hybridomas. Journal of Immunological Methods 39, 285-308.

- Goding, J.W. (1986). In: Monoclonal Antibodies : Principles and Practice. London Academic Press 2nd Edition.
- Goka, A.K.J. & Farthing, M.J.G. (1987). The use of 3,3',5,5'-tetramethylbenzidine as a peroxidase substrate in microplate enzyme-linked immunosorbent assay. Journal of Immunoassay 8, 29-41.
- Goodwin, P.H., DeVay, J.E. & Meredith, C.P. (1988). Physiological response of Vitis vinifera cv. "Chardonnay" to infection by Pierce's disease bacterium. Physiological and Molecular Plant Pathology. 32, 17-32.
- Goto, M. (1972). Interrelationship between colony type, phage susceptibility and virulence in Xanthomonas campestris. Journal of Applied Bacteriology 35 505-515.
- Gough, N.M. & Cory, S. (1986). In: Handbook of Experimental Immunology. D.M. Weir, L.A. Herzenberg, C. Blackwell & L.A. Herzenberg (Editors). Blackwell Scientific Publications, Oxford. 4th Edition.
- Gram, C. (1884). Fortschr med. ii, 185-190.
- Griffiths, B. (1986). Scaling-up of animal cell cultures. In: Animal Cell Culture; A Practical Approach pp33-70. R.I. Freshney (Ed.). Oxford. IRL Press.
- Grogan, R.G. & Kendrick, J.B. (1953). Seed transmission, mode of over-wintering and spread of bacterial canker of tomato caused by Corynebacterium michiganense. Phytopathology 43, 473 (Abstr.).
- Gross, D.C. & Cody, Y.S. (1985). Mechanisms of plant pathogenesis by Pseudomonas species. Canadian Journal of Microbiology 31, 403-410.
- Gross, D.C., Cody, Y.S., Proebsting E.L., Radamaker, G.K. & Sputts, R.A. (1984). Ecotypes and pathogenicity of ice-nucleation active P. syringae isolated from deciduous fruit tree orchards. Phytopathology 74, 241-248.

- Guzman, M.L., Luna, J. & Victoria, J.I. (1989) The latex agglutination test as serological assay for ratoon stunting disease. In Press.
- Hadfield, S.G., Lane, A. & McIllmurray, M.B. (1987). A novel coloured latex test for the detection and identification of more than one antigen. Journal of Immunological Methods 97, 153-158.
- Halk, E.L. & De Boer, S.H. (1985). Monoclonal antibodies in plant-disease research. Annual Review of Phytopathology 23, 321-350.
- Halverson, L.J. & Stacey, G. (1986). Signal exchange in Plant-Microbe Interactions. Microbiological Reviews 50, 193-225.
- Hammerschmidt, R., Lamport, D.T.A. & Muldon, E.R. (1984). Cell wall hydroxyproline enhancement and lignin deposition as an early event in the resistance of cucumber Cladosporium cucumerinum. Physiological Plant Pathology 24, 43-47.
- Hancock, I. & Poxton, I. (1988). Bacterial cell surface techniques. Wiley Chichester. 329pp.
- Hancock, R.E.W., Wieczoreck A.A., Mutharia, L.M. & Poole, K. (1982). Monoclonal antibodies against Pseudomonas aeruginosa outer membrane antigens : Isolation and characterization. Infection and Immunity 37, 166-171.
- Hawkes, R., Niday, E. & Gordon, J. (1982). A dot-immunobinding assay for monoclonal and other antibodies. Analytical Biochemistry 119, 142-147.
- Hayward, A.C. (1964). Characteristics of Pseudomonas solanacearum. Journal of Applied Bacteriology 27, 265-277.
- Hayward, A.C. (1985), Bacterial wilt caused by Pseudomonas solanacearum in Asia and Australia: an overview. In: Bacterial Wilt Disease in Asia and the South Pacific. G.J. Persley (Editor). ACIAR Proceedings No. 13. pp15-24.

- Hayward, A.C. & Waterston, J.M. (1964). Corynebacterium insidiosum. In: CMI Descriptions of Pathogenic Fungi and Bacteria. 13 Commonwealth Mycological Institute, Kew.
- Hayward, A.C. & Waterston, J.M. (1965). Corynebacterium flaccumfaciens. In: CMI Descriptions of Pathogenic Fungi and Bacteria. 43 Commonwealth Mycological Institute, Kew.
- Hayward, A.C. (1986). Bacterial Wilt caused by P. solanacearum in Asia and Australia: an overview. In: Bacterial Wilt Disease in Asia and the South Pacific: Proceedings of an international workshop held at PCARRD, Los Banos, Philippines, 8-10 October, 1985. ACIAR Proceedings no.13. pp15-24
- Hendrick, C.A. & Sequeira, L. (1984). LPS defective mutants of the wilt pathogen P. solanacearum. Applied and Environmental Microbiology 48, 94-101.
- Heusser, C.H., Stocker, J.W. & Gisler R.H. (1981). Methods for binding cells to plastic: Application to solid phase immunoassays for cell-surface antigens. Methods In Enzymology 73, 406-418.
- Hiatt, A., Cafferkey, R. & Bowdish, K. (1989). Production of antibodies in transgenic plants. Nature 342, 76-78.
- Hidir Sastraatmadja (1973). Penyakit-penyakit cengkeh di Indonesia dan usaha penanggu-langnnya di lapangan. In: Workshop of Direktorat Jenderal Perkebunan. Ciawi, Bogor, Indonesia. 134-146.
- Hirano, S.S. & Upper, C.D. (1983). Ecology and Epidemiology of foliar bacterial plant pathogens. Annual Review Phytopathology 21, 243-269.
- Hitchcock, P.J. & Brown, T.M. (1983). Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver stained polyacrylamide gels. Journal of Bacteriology 154, 269-277.

- Hobot, J.A., Carlemalm, E., Villiger, W. & Kellenberger, E. (1984). Periplasmic gel. New concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. Journal of Bacteriology 160, 143-52.
- Hofstra, H. & Dankert, J. (1979). Antigenic cross-reactivity of major outer membrane proteins in Enterobacteriaceae species. Journal of General Microbiology 111, 293-302.
- Holme, T. & Gustafsson, B. (1985). Monoclonal antibodies against group- and type-specific antigens of Vibrio cholerae O : 1. In: Monoclonal Antibodies Against Bacteria. Vol. I A.J.L. Macario & E.C.de Macario (Eds.). London. Academic Press. pp 167-189.
- Honjo, T. (1983). Immunoglobulin genes. Annual Review Immunology 1, 499-528.
- Hopkins, D.L. (1977). Diseases caused by leafhopper borne rickettsia-like bacteria. Annual Review of Phytopathology 17, 277-294.
- Hopkins, D.L. (1981). Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. Phytopathology 71, 415-418.
- Hopkins, D.L. (1989). Xylella fastidiosa: xylem-limited bacterial pathogen of plants. Annual Review of Phytopathology 27, 271-290.
- Horitaba, K., & Harris, A.W. (1970). Mouse myelomas and lymphomas in culture. Experimental Cell Research 60, 61-77.
- Houghton, A.N. (1988). Building a better monoclonal antibody. Immunology Today 9, 265-267.
- Huber, R., Deisenhofer, J., Colman, P.M., Matsushima, M., & Palm, W. (1976). Crystallographic structure studies of an IgG molecule and an Fc fragment. Nature 264, 415-420.
- Hudson, L. & Hay, F.C. (1980). Practical Immunology 2nd Ed. Oxford. Blackwell Scientific Publications. 359pp

- Hunt, P., Bennett, C.P.A. & Ariful Asman, (1985).
Suppression of Sumatra disease symptoms in cloves
treated with antibiotics. Plant Pathology 34, 495-
501.
- Hunt, P., Bennett, C.P.A., Hilma Syamsu & Endang Nurwenda
(1987). Sumatra disease in cloves induced by a
xylem-limited bacterium following mechanical
inoculation. Plant Pathology 36, 154-163.
- Husain, A. & Kelman, A. (1958). The role of pectic and
cellulolytic enzymes in pathogenesis by p.
solanacearum. Phytopathology 48, 377-386.
- Inouye, M., Shaw, J. & Shen, C. (1972). The assembly of a
structural lipoprotein in the envelope of
Escherichia coli. Journal of Biological Chemistry
247, 8154-8159.
- Janse, J.M. (1892). Nota over eene ziekte der tabak op
Sumatra. Teysmannia 3, 653-662.
- Johnson, G.D., Holborow, E.J. & Dorling, J. (1978).
Immunofluorescence and immunoenzyme techniques.
Chapter 15 in: Handbook of Experimental Immunology.
Vol 1. Immunochemistry D.M. Weir (Ed.). Oxford.
Blackwell Scientific Publications.
- Johnson, H.A. & Powell, N.T. (1969). Influence of root
knot nematodes on bacterial wilt development in
flue-cured tobacco. Phytopathology 59, 486-491.
- Johnstone, A. & Thorpe, R. (1987). Immunochemistry in
Practice (2nd edition). Blackwell Scientific
Publications, Oxford.
- Jones, P.T., Dear, P.H., Foote, J., Neuberger, M.S. &
Winter, G. (1986). Replacing the complementarity
determining regions in a human antibody with those
from a mouse. Nature 321, 522-555.
- Kabat, E.A. (1976). Structural Concepts in Immunology and
Immunochemistry. Holt Rinehart. 2nd ed.
- Karsten, U. & Rudolph, M. (1985). Monoclonal antibodies
against tumour-associated antigens: mycoplasma as a
major technical obstacle and its possible

- circumvention by azaserine selection medium. Archiv für Geschwulstforschung 55, 305-310.
- Kauffmann, F. (1966). The bacteriology of Enterobacteriaceae. Monksgaard, Copenhagen. 400pp.
- Kawaiji, H., Mizuno, T. & Mizushima, S. (1979). Influence of molecular size and osmolarity of sugars and dextrans on the synthesis of outer membrane proteins 0.8 - 0.9 of E. coli K-12. Journal of Bacteriology 140, 843-847.
- Kelman, A. (1953). The bacterial wilt caused by Pseudomonas solanacearum. North Carolina Agricultural Experimental Station Technical Bulletin 99, 194pp.
- Kelman, A. (1954). The relationship of pathogenicity of P. solanacearum to colony appearance on a tetrazolium medium. Phytopathology 44, 693-695.
- Kelman, A. & Sequeira, L. (1965). Root-to-root spread of P. solanacearum. Phytopathology 55, 304-309.
- Kelman, A. & Cowling, E.B. (1965). Cellulose of P. solanacearum in relation to pathogenesis. Phytopathology 55, 148-155.
- Kelman, A. & Sequeira, L. (1972). Resistance in plants to bacteria. Proceedings of the Royal Society 181, 247-266.
- Kemeny, D.M. & Chantler, S. (1988). An introduction to ELISA. In: Elisa and Other Solid Phase Assays D.M. Kemeny & S.J. Challacombe (Eds.). John Wiley & Sons. Chichester. pp 1-29.
- Kennett, R.H., Davis, K.A., Tung, A.S., & Klinman, N.R. (1978). Hybrid plasmacytoma production: fusion with adult spleen cells, monoclonal spleen fragments, neonatal spleen cells and human spleen cells. Current Topics in Microbiology & Immunology 81, 77-91.
- Kerr, A. (1980). Biological control of crown gall through production of agrocin 84. Plant Disease 64, 25-30.

- Köhler, G. & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predetermined specificity. Nature 256, 495-497.
- Köhler, G. & Milstein, C. (1976). Derivation of specific antibody-producing tissue culture and tumour lines by cell fusion. European Journal of Immunology 6, 511-519.
- Kronick, M.N. (1986). The use of phycobiliproteins as fluorescent labels in immunoassay. Journal of Immunological Methods 92, 1-13.
- Kronvall, G. (1973). A rapid slide-agglutination method for typing pneumococci by means of specific antibody absorbed to Protein A-containing Staphylococci. Journal of Medical Microbiology 6, 187-190.
- Kuhara, S. (1978). Present epidemic status and control of the citrus canker disease X. citri in Japan. Review of Plant Protection Research 11, 132-142.
- Lane, D. & Koprowski, H. (1982). Molecular recognition and future of monoclonal antibodies. Nature 296 200-202.
- Lelliott, R.A., Billing, E. & Hayward, A.C. (1966). A determinative scheme for the fluorescent plant pathogenic pseudomonads. Journal of Applied Bacteriology 29, 470-489.
- Lelliott, R.A. & Stead, D.E. (1987). Methods for the diagnosis of bacterial diseases of plants. In: Methods in Plant Pathology (Volume 2). (Editor T.Preece). Blackwell Scientific Publications. 216pp.
- Lin, C.P. & Chen, T.A. (1986). Comparison of monoclonal antibodies and polyclonal antibodies in detection of the aster yellows mycoplasmalike organism. Phytopathology 76, 45-50.
- Lin, C.P., Chen, T.A., Wells, J.M. & van der Zwet, T. (1987). Identification and detection of Erwinia amylovora with monoclonal antibodies. Phytopathology 77, 376-380.

- Littlefield, S.W. (1964). Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145 709-710.
- López, M.M., Cambra, M., Aramburu, J.M. & Bolinches J. (1987). Problems of detecting phytopathogenic bacteria by ELISA. Bulletin OEPP/EPPO Bulletin 17, 113-117.
- Lowe, A.D., Lennox, E.S. & Voak, D. (1984). A new monoclonal anti-A: culture supernatants with the performance of hyperimmune human reagents. Vox Sanguinis 46, 29-35.
- Luderitz, O., Galanos, C., Risse, H.J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthausen, H., Wheat, R., Westphal, O. & Schlosshardt, J. (1966). Structural relationships of Salmonella O and R antigens. Annals of the New York Academy of Science 133, 349-374.
- Luderitz, O., Galanos, C., Lehmann, V., Nurminen, M., Rietschel, E.T., Rosenfelder, G., Simon, M. & Westphal, O. (1973). Lipid A: chemical structure and biological activity. Journal of Infectious Diseases 128, (supp.) S17-S29.
- Lugtenberg, B. & van Alphen, L. (1983). Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. Biochemica et Biophysica Acta 737, 51-115.
- Lund, B.M. (1979). Bacterial soft-rot of potatoes. In: Plant Pathogens D.W. Lovelock (Editor) Society for Applied Biology Technical Series no. 12. London & New York Academic Press pp19-49.
- Lyon, F.M. & Wood, R.K.S. (1975). Production of phaseollin and related compounds in bean leaves inoculated with Pseudomonas spp. Physiological Plant Pathology 70, 406-409.

- McCullough, K.C. & Parkinson, D. (1984). The standardization of a "spot-test" ELISA for the rapid screening of sera and hybridoma cell products II. The determination of binding capacity, binding ratio and coefficient of variation of different ELISA plates in sandwich and indirect ELISA. Journal of Biological Standardization 12, 75-86.
- Macario, A.J.L. & Conway de Macario, E. (1982). The immunology of methanogens: A new development in microbial biotechnology. Immunology Today 3, 279-284.
- Macario, A.J.L. & Conway de Macario, E. (1984). Antibacterial monoclonal antibodies and the dawn of a new era in the control of infection. Survey and Synthesis Pathology Research 3, 119-130.
- Macario, A.J.L. & Conway de Macario, E. (Eds.) (1985a). Monoclonal Antibodies Against Bacteria. Vol. I. London. Academic Press.
- Macario, A.J.L. & Conway de Macario, E. (Eds.) (1985b). Monoclonal Antibodies Against Bacteria. Vol. I. London. Academic Press.
- Machmud, M. (1985). Bacterial wilt in Indonesia. In: Bacterial Wilt Disease in Asia and the South Pacific. G.J. Persley (Editor). ACIAR Proceedings no. 13. pp30-34.
- Machmud, M. (1987). New hosts for Pseudomonas solanacearum. Bacterial Wilt Newsletter. 2, 8.
- Male, D., Champion, B., & Cooke, A. (1987). Advanced Immunology Gower Medical Publishing. London.
- Malin, E., Belden, E.L. & Roth, D. (1985). Evaluation of the radioimmunoassay, indirect enzyme linked immunoabsorbent assay, and dot blot assay for the identification of Xanthomonas campestris pv. phaseoli. Canadian Journal of Plant Pathology 7, 217-222.
- Mansfield, J.W., Hargreaves, J.A. & Boyle, F.C. (1974). Phytoalexin production by live cells in broad bean

- leaves infected with Botrytis cinerea. Nature 252, 316-317.
- Martin, J.T. (1964). Role of cuticle in the defence against plant disease. Annual Review of Phytopathology 2, 81-100.
- Martin, R.R. (1987). The use of monoclonal antibodies for virus detection. Canadian Journal of Plant Pathology 9, 177-181.
- Martin-Gros, G., Iskra, M.L., Garnier, M., Gandar, J. & Bové J.M. (1987). Production of monoclonal antibodies against phloem-limited prokaryotes of plants: a general procedure using extracts from infected periwinkles as immunogen. Annals Institut Pasteur/Microbiologie 138, 625-637.
- Max, E.E., Serdman, J.G. & Leder, P. (1979). Sequences of five potential recombination sites encoded close to an immunoglobulin K constant region gene. Proceedings of the National Academy of Science, U.S.A. 76 3450-3454.
- Mayer, H. & Weckesser, J. (1984). In: "Chemistry of Endotoxin" E.T. Rietschel, Editor. Volume 1. Elsevier North Holland, Amsterdam.
- Mayer, H., Tharanathan, R.N., & Weckesser, J. (1985). Analysis of lipopolysaccharides of Gram-negative bacteria. Methods in Microbiology 18, 157-207.
- McMichael, A.J. & Fabre, J.W. (Eds.). (1982). Monoclonal antibodies in clinical medicine. London. Academic Press.
- Mew, T.W. & Ho, W.C. (1976). Varietal resistance to bacterial wilt in tomato. Plant Disease Reporter 60 264-268.
- Miller, H.J. (1984). A method for the detection of latent ringrot in potatoes by immunofluorescence microscopy. Potato Research 27, 33-42.
- Milstein, C. (1986). Overview: Monoclonal antibodies. In: Handbook of Experimental Immunology Chapter 107). D.M. Weir (Editor).

- Milstein, C. & Cuello, A.C. (1984). Hybrid hybridomas and the production of bispecific monoclonal antibodies. Immunology Today 5, 299-304.
- Milstein, C., Wright, B. & Cuello, A.C. (1983). The discrepancy between the cross-reactivity of a monoclonal antibody to serotonin and its immunohistochemical specificity. Molecular Immunology 20, 113-117.
- Mircetich, S.M., Lowe, S.K., Moller, W.J. & Nyland, G. (1976). Etiology of almond leaf scorch disease and transmission of the causal agent. Phytopathology 66 17-24.
- Mitchell, R.E. (1981). Structure: Bacteria. In: Toxins in Plant Disease R.D. Durbin (Editor). Academic Press, New York. pp259-293.
- Mizuno T., Chou, M.Y. & Inouye, M. (1983). A comparative study on the genes for three porins of the Escherichia coli outer membrane. Journal of Biological Chemistry 258, 6932-6940.
- Moffett, M.L., Wood, B.A. & Hayward, A.C. (1981). Seed and soil: sources of inoculum for the colonization of the foliage of solanaceous hosts by P. solanacearum. Annals of Applied Biology 98, 403-411.
- Morrison, S.L., Johnson, M.J., Herzenberg, L.A. & Oi, V.T. (1984). Chimeric human antibody molecules: mouse antigen binding domains with human constant region domains Proceedings of the National Academy of Sciences USA 81, 6851-6855.
- Mosmann, T.R., Gallatin, M. & Langenecker, B.M. (1980). Alteration of apparent specificity of monoclonal (hybridoma) antibodies recognising polymorphic histocompatibility and blood group determinants. Journal of Immunology 125, 1152-1156.
- Moyer, J.W. & Echandi, E. (1986). Serological detection and identification of Streptomyces ipomoea. Plant Disease 70, 516-518.

- Murray, R.G.E., Stead, P. & Elson, H.E. (1965). The localization of the mucopeptide in sections of the cell wall of Escherichia coli and other gram-negative bacteria. Canadian Journal of Microbiology 11, 547-560.
- Mutharia, L.M., Lam, J.S. & Hancock, R.E.W. (1985). Use of monoclonal antibodies in the study of common antigens of Gram-negative bacteria. In: Monoclonal Antibodies Against Bacteria. Vol. II A.J.L. Macario & E.C.de Macario (Eds.). London. Academic Press. pp 131-142.
- Nakae, T. (1976). Identification of the major outer membrane protein of E. coli that produces transmembrane channels in reconstituted vesicle membranes. Biochemistry and Biophysics Research Communications 71, 877-884.
- Nelson, P.E. & Dickey, R.S. (1970). Histopathology of Plants Infected With Vascular Bacterial Pathogens. Annual Review of Phytopathology 8 259-280.
- Nester, E.W. & Kosuge, T. (1981). Plasmids specifying plant hyperplasias. Annual Review of Microbiology 35 531-565.
- Neuberger, M.S., Williams, G.T. & Fox, R.O. (1984). Recombinant antibodies possessing novel effector functions. Nature 312, 604-608.
- Neuberger, M.S. (1985). Making novel antibodies by expressing transfected Ig genes. Trends in Biological Sciences 10, 347-349.
- Nicas, T.L. & Hancock, R.E.W. (1980). Outer membrane protein H1 of Pseudomonas aeruginosa: involvement in adaptive and mutational resistance to EDTA, polymyxin B and gentamicin. Journal of Bacteriology 143, 872-878.
- Nikaido, H. (1970). Lipopolysaccharide in the taxonomy of Enterobacteriaceae. International Journal of Systematic Bacteriology 20, 383-406.

- Nikaido, H. & Nakae, T. (1979). The outer membrane of gram-negative bacteria. Advances in Microbiological Physiology 20, 163-250.
- Nikaido, H. & Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. Microbiological Reviews 49, 1-32.
- Nossa, G.J.V., & Lederberg, J. (1958). Antibody production by single cells. Nature 181 1419-1420.
- Nuttall, V.W. & Jasmin, J.J. (1958). The inheritance of resistance to bacterial wilt (Erwinia tracheiphila E.F. Smith, Holland) in cucumber. Canadian Journal of Plant Science 38 401-604.
- Oellermann, R.A. & Pillay, D. (1987). Immunological methods for the detection of a coryneform isolated from ratoon stunted sugarcane. Journal of Phytopathology 118, 59-67.
- Okada, Y. (1962). Analysis of giant polynuclear cell formation caused by HVJ virus from Ehrlich's ascites tumour cells. I. Microscopic observation of giant polynuclear cell formation. Experimental Cell Research 26, 98-107.
- Osborn, M.J. (1963). Studies on the Gram-negative cell wall. 1. Evidence for the role of 2-keto-3 deoxyoctonate in the LPS of Salmonella typhimurium. Proceedings of the National Academy of Science, USA. 50, 499-506.
- Ouchterlony, O. & Nilsson, L.A. (1978). Immunodiffusion and immunoelectrophoresis. Chapter 19 in: Handbook of Experimental Immunology. Vol 1. Immunochemistry D.M. Weir (Ed.). Oxford. Blackwell Scientific Publications.
- Ouellette, G.B. & Benhamou, N. (1987). Use of monoclonal antibodies to detect molecules of fungal plant pathogens. Canadian Journal of Plant Pathology 9, 167-176.
- Palva, E.T. (1978) Major OM protein in S. typhimurium induced by maltose. Journal of Bacteriology 136 286-

- Palva, E.T. & Randall, L.L., (1978) Arrangement of protein I in E. coli outer membrane: cross-linking study. Journal of Bacteriology 133 271-286.
- Palva, E.T. & Westerman, P. (1979) Arrangement of the maltose - inducible major outer membrane proteins, the bacteriophage receptor in E. coli and the 44K protein in S. typhimurium. FEBS Letters 91 77-80.
- Palleroni, N.J. & Doudoroff, M. (1971). Phenotypic characterization and deoxyribonucleic acid homologies of Pseudomonas solanacearum. Journal of Bacteriology 107, 690-696.
- Panopoulos, N.J. & Schroth, M.N. (1974). Role of flagellar motility in the invasion of bean leaves by P. phaseolicola. Phytopathology 64 1389-1397.
- Paranchych, W. & Frost, L.S. (1988). The Physiology and Biochemistry of Pili. Advances in Microbial Physiology 29 53-114.
- Parsons, G.H. Jr. (1981). Antibody-coated plastic tubes in radioimmunoassay. Methods in Enzymology 73, 224-239.
- Pate, J.S. (1976). Nutrients and metabolites of fluids recovered from xylem and phloem: significance in relation to long distance transport in plants. In: Transport and Transfer Processes in Plants I.F. Wordlaw & J.B. Passiours (Editors) New York Academic Press. pp253-281.
- Pegg, G.F. (1974). Verticillium diseases. Review of Plant Pathology 53 157-182.
- Pegg, G.F. & Cronshaw, D.K. (1976). The relationship of in vitro to in vivo ethylene production in P. solanacearum infection of tomato. Physiological Plant Pathology 9 145-154.
- Pegg, G.F. (1985). Life in a black hole - the micro-environment of the vascular pathogen. Transactions of the British Mycological Society 85 1-20.

- Perombelon, M.C.M. & Kelman, A. (1980). Ecology of the soft rot Erwinias. Annual Review of Phytopathology 18 361-387.
- Perry, M.B., MacLean, L. & Griffith, D.W. (1986). Structure of the O-chain polysaccharide of the phenol-phase soluble lipopolysaccharide of Escherichia coli O:157:H7. Canadian Journal of Biochemistry and Cell Biology 64, 21-28.
- Persley, G.J. (1986a). Ecology of P. solanacearum, the causal agent of bacterial wilt. In: Bacterial Wilt Disease in Asia and the South Pacific: Proceedings of an international workshop held at PCARRD, Los Banos, Philippines, 8-10 October, 1985. ACIAR Proceedings no.13. pp71-76 145 pp.
- Persley, G.J. (Ed.) (1986b). Bacterial Wilt Disease in Asia and the South Pacific: Proceedings of an international workshop held at PCARRD, Los Banos, Philippines, 8-10 October, 1985. ACIAR Proceedings no.13. 145 pp.
- Pillay, D. & Oellermann, R.A. (1988). Enzyme-Linked Immunosorbent Assays (ELISA) of a coryneform isolated from ratoon stunted sugarcane. Journal of Phytopathology 121, 166-174.
- Pine, T.S., Grogan, R.G. & Hewitt, W.B. (1955). Pathological anatomy of bacterial canker of young tomato plants. Phytopathology 45 267-271.
- Polin, R.A. (1984). Monoclonal antibodies against microorganisms. European Journal of Clinical Microbiology 3, 387-398.
- Polin, R.A. & Kennet, R. (1980). Use of monoclonal antibodies in an enzyme immunoassay for rapid identification of group B streptococcus types II and III. Journal of Clinical Microbiology 11, 332-336.
- Pool, P.A., Eden-Green, S.J. & Muhammad, M.T. (1986). Variation in clove (Syzygium aromaticum) germplasm in the Moluccan islands. Euphytica 35, 149-159.

- Poole, K. & Hancock, R.E.W. (1986). Phosphate-starvation-induced outer membrane proteins of members of the families Enterobacteriaceae and Pseudomonadaceae: demonstration of immunological x-reactivity with an antiserum specific for porin protein P of P. aeruginosa. Journal of Bacteriology 165 987-993.
- Porter, R.R. (1967). The structure of antibodies. Scientific American 217 81-90.
- Potter, M. & Boyce, C.R. (1962). Induction of plasma cell neoplasms in strain BALB/c mice with mineral oil adjuvants. Nature 193 1086-1087.
- Powell, C.A. (1987). Detection of three plant viruses by dot-immunobinding assay. Phytopathology 77, 306-309
- Poxton, I.R., Bell, G.T. & Barclay, G.R. (1985). The association on SDS-polyacrylamide gels of lipopolysaccharide and outer membrane proteins of Pseudomonas aeruginosa as revealed by monoclonal antibodies and Western blotting. Federation of European Microbiological Societies Microbiology Letters 27, 247-251.
- Purcell, A.H. (1979). Leafhopper vectors of xylem-borne plant pathogens In: Leafhopper vectors and Plant Disease Agents K. Maramorosch & K. Harris (Editors). New York Academic Press. pp603-627.
- Purcell, A.H. & Finlay, A.H. (1979). Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. Phytopathology 69 393-395.
- Purcell, A.H., Finlay, A.H. & McLean, D.L. (1979). Pierce's disease bacterium: Mechanism of transmission by leafhopper vectors. Science 206, 839-841.
- Raju, B.C., Wells, J.M., Nyland, G., Bransky, R.H. & Lowe, S.K. (1982). Plum leaf scald: isolation, culture and pathogenicity of the causal agent. Phytopathology 72 1460-1466.

- Raju, B.C. & Wells, J.M. (1986). Diseases caused by fastidious xylem-limited bacteria and strategies for management. Plant Disease 70, 182-186.
- Rau, M.U.B., Sohi, H.S. & Tikoo, S.K. (1975). Reaction of wilt-resistant tomato varieties and lines to P. solanacearum in India. Plant Disease Reporter 59, 734-736.
- Reeves, G. (1987). Lecture notes in immunology. Oxford. Blackwell. 197pp.
- Riechmann, L., Clark, M., Waldman, H. & Winter, G. (1988). Reshaping human antibodies for therapy. Nature 332, 323-327.
- Ries, S.M. & Strobel, G.A. (1972). Biological properties and pathological role of a phytotoxic glycopeptide from Corynebacterium insidiosum. Physiological Plant Pathology 2 133-142.
- Ringertz, N.R. & Savage, R.E. (1976). Cell Hybrids. Academic Press, New York.
- Roberts, D.P., Denny, T.P. & Schell, M.A. (1988). Cloning of the egl gene of P. solanacearum and analysis of its role in phytopathogenicity. Journal of Bacteriology 170, 1445-1451.
- Roberts, P. (1980). Problems encountered during immunofluorescent diagnosis of fireblight. Plant Pathology 29, 93-97.
- Roberts, S.J., Eden-Green, S.J., Jones, P. & Ambler, D.J. (1990). Pseudomonas syzygii, sp. nov., the cause of Sumatra disease of cloves. Systematic and Applied Microbiology 13, 34-43.
- da Rocha, A., Ohki, S.T. & Hiruki, C. (1986). Detection of mycoplasma-like organisms in situ by indirect immunofluorescence microscopy. Phytopathology 76, 864-868.
- Rogers, H.J., Perkins, H.R. & Ward J.B. (1980). In: Microbiology Cell walls and Membranes. Chapman & Hall, London. pp105-106.

- Roitt, I.M. (1988). Essential Immunology. Blackwell. Oxford.
- Ryan, J.M. & Conrad, H.E. (1974). Structural heterogeneity in the lipopolysaccharide of Salmonella newington. Archives of Biochemistry and Biophysics 162, 530-535.
- Sakano, H., Hippi, K., Heinrich, G. & Tonegaura, S. (1979). Sequences at the somatic recombination sites of immunoglobulin light-chain genes. Nature 280 288-294.
- Sakano, H., Kurosaura, Y., Weigert, M. & Tonegaura, S. (1981). Identification and nucleotide sequence of a diversity DNA segment (D) of immunoglobulin heavy-chain genes. Nature 290 562-565.
- Salton, M.R.J. (1961). The anatomy of the bacterial surface. Bacteriological Reviews 25, 77-99.
- Salton, M.R.J. (1987). Bacterial membrane proteins. Microbiological Sciences 4, 100-105.
- Schaad, N.W. (1978). Use of direct and indirect immunofluorescence tests for identification of Xanthomonas campestris. Phytopathology 68, 249-252.
- Schaad, N.W. (1979). Serological identification of plant pathogenic bacteria. Annual Review of Phytopathology 17, 123-147.
- Schell, M.A., Roberts, D.P. & Denny, T.P. (1988). Analysis of the P. solanacearum polygalacturonase encoded by pglA and its involvement in phytopathogenicity. Journal of Bacteriology 170 4501-4508.
- Schleifer, K.H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Review 36, 407-477.
- Schleifer, K.H. & Stackebrandt, E. (1983). Molecular systematics of prokaryotes. Annual Review of Microbiology 37, 143-187.
- Schmidt, J. (1978). Microscopic study of early stages of

- infection by P. solanacearum on in vitro grown tomato seedlings. Proceedings 4th International Conference on Plant Pathogenic Bacteria, Angers 1978 pp841-856.
- Schroth, M.N., Thomson, S.V. & Hildebrand, D.C. (1974). Epidemiology and control of fire blight. Annual Review of Phytopathology 12, 389-412.
- Sequeira, L. (1958). Bacterial wilt of bananas. Dissemination of the pathogen and control of the disease. Phytopathology 48, 64-69.
- Sequeira, L. & Kelman, A. (1962). The accumulation of growth substances in plants infected by P. solanacearum. Phytopathology 52, 439-448.
- Sequeira, L. (1965). Origin of indoleacetic acid in tobacco plants infected by P. solanacearum. Phytopathology 55, 1232-1236.
- Sequeira, L. & Rowe, P.R. (1969). Selection and utilization of Solarium phureja clones with high resistance to different strains of P. solanacearum. American Potato Journal 46, 451-462.
- Sequeira, L. & Graham, T.L. (1977). Agglutination of avirulent strains of P. solanacearum by potato lectin. Physiological Plant Pathology 11, 43-54.
- Sequeira, L. (1979). In: Host - Parasite Interfaces B.B. Nickol (Editor). Academic Press, Orlando & London. pp71-84.
- Sherald, J.L., Hearon, S.S., Kostka, S.J. & Morgan, D.L. (1983). Sycamore leaf scorch: culture and pathogenicity of fastidious xylem-limited bacteria from scorch-affected trees. Plant Disease 67, 849-852.
- Sherman, D.M., Acres, S.D., Sadowski, P.L., Springer, J.A., Bray, B., Raybould, T.J.G. & Muscoplat, C.C. (1983). Protection of calves against fatal enteric coli-bacillosis by orally administered Escherichia coli K99-specific monoclonal antibody. Infection and Immunity 42, 653-658.

- Shimizu, A., Takahashi, N., Yaoita, Y. and Honjo, T. (1982). Organization of constant region gene family of the mouse immunoglobulin heavy chain. Cell 28, 499-506.
- Shimizu, A. & Honjo, T. (1984). Immunoglobulin class switching. Cell 36, 801-807.
- Sinha, R.C. & Chiykowski, L.N. (1984). Purification and serological detection of mycoplasmalike organisms from plants affected by peach eastern X-disease. Canadian Journal of Plant Pathology 6, 200-205.
- Slack, S.A., Sanford, H.A. & Manzer, F.E. (1979). The latex agglutination test as a rapid serological assay for Corynebacterium sepedonicum. American Potato Journal 56, 441-446.
- Sleytr, U.B. & Messner, P. (1983). Crystalline surface layers on bacteria. Annual Review of Microbiology 37 311-339.
- Smit, J., Kamio, Y. & Nikaido, H. (1975). Outer membrane of Salmonella typhimurium: chemical analysis and freeze- fracture studies with LPS mutants. Journal of Bacteriology 124, 942-958.
- Smith, E., Roberts, K., Butcher, G.W. & Galfré, G. (1984). Monoclonal antibody screening: Two methods using antigens immobilized on nitrocellulose. Analytical Biochemistry 138, 119-124.
- Smith, J.J. & Mansfield, J.W. (1982). Ultrastructure of interactions between pseudomonads and oat leaves. Physiological Plant Pathology. 21, 259-266.
- Smyth, C.J., Siegel, J., Salton, M.R.J. & Owen, P. (1978) Immunochemical analysis of inner and outer membranes of Escherichia coli by crossed immunoelectrophoresis. Journal of Bacteriology. 133, 306-319.
- Soini, E. & Hemmila, I. (1979). Fluoroimmunoassay: present status and key problems. Clinical Chemistry 25, 353-361.
- Sonada, R.M. & Augustine, J. (1978). Reaction of

- bacterial wilt , resistant tomato lines to P. solanacearum in Florida. Plant Disease Reporter 62, 464-466.
- Stack, J.P., Mount, M.S., Berman, P.M. & Hubbard, J.P. (1980). Pectic enzyme complex from Erwinia carotovora. A model for degradation and assimilation of host pectic fractions. Phytopathology 70 267-272.
- Stall, R.E. & Seymour, C.P. (1983). Canker, a threat to citrus in the Gulf-Coast States. Plant Disease 67, 581-585.
- Stapp, C. (1961). Bacterial Plant Pathogens Oxford University Press, London 292pp.
- Starr, M.P., Stolp, H., Truper, H.G., Balows, A. & Schlegel, H.G. (Eds.). (1981). The Prokaryotes. Volume I. Berlin. Springer-Verlag. 1102pp.
- Staub, T. & Williams, P.H. (1972). Factors influencing black rot lesion development in resistant and susceptible cabbage. Phytopathology 62 722-728.
- Stemmer, P. & Sequeira, L. (1981). Pili of plant pathogenic bacteria. Phytopathology 71, 906 (Abstr.).
- Stock, J.B., Rauch, B. & Rosemans, S. (1977). Periplasmic space in Salmonella typhimurium and Escherichia coli. Journal of Biological Chemistry 252, 7850-7861.
- Strange, R.E. & Dark, F.A. (1956). An unidentified amino-sugar present in cell walls and spores of various bacteria. Nature 177, 186-188.
- Strobel, G.A. & Lanier, G.N. (1981). Dutch Elm Disease. Scientific American 245 56-66.
- Sutherland, I.W. (1977). In: Surface Carbohydrates of the Prokaryotic Cell. I.W. Sutherland (Editor) Academic, New York. pp27-96.
- Sutula C.L., Gillett, J.M., Morrissey S.M. & Ramsdell D.C. (1986). Interpreting ELISA data and establishing the positive-negative threshold. Plant

Disease 70, 722-726.

- Taussig, M.J. (1988). Molecular genetics of immunoglobulins. Immunology supplement 1 7-16.
- Thaveechai, N. & Schaad, N.W. (1984). Comparison of different immunogen preparations for serological identification of Xanthomonas campestris pv. campestris. Phytopathology 74, 1065-1070.
- Thompson, R.J. & Jackson, A.P. (1984). Cyclic complexes and high avidity antibodies. Trends in Biological Sciences 9, 1-3.
- Thurston, H.D. (1976). Resistance to bacterial wilt (P. solanacearum). In: Planning conference and workshop on the ecology and control of bacterial wilt caused by P. solanacearum. L. Sequeira & A. Kelman (Editors). Raleigh North Carolina N.C. State University, 58-62.
- Tiffin, A.I. (1987). Monoclonal antibodies and their use in microbiology. Journal of Applied Bacteriology Symposium Supplement, 127-139.
- Timmer, L.W., Brlansky, R.H., Lee, R.F. & Raju, B.C. (1983). A fastidious, xylem-limited bacterium infecting ragweed. Phytopathology 73 975-979.
- Tjahyono, B. & Eden-Green, S.J. (1988). Blood disease of bananas in Indonesia. In: Proceedings of the 5th International Congress of Plant Pathology. Kyoto, Japan, 1988. p389.
- Tjissen, P. (1985). Practice and theory of enzyme immunoassays. In: Laboratory Techniques in Biochemistry and Molecular Biology Volume 15. R.H. Burdon & P.H. van Knippenberg (Editors). Elsevier, Amsterdam. 550pp.
- Towbin, H. & Gordon, J. (1984). Immunoblotting and dot immunobinding - current status and outlook. Journal of Immunological Methods 72, 313-340.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets:

- procedure and some applications. Proceedings of the National Academy of Science, USA 76, 4350-4354.
- Tsai, C.M. & Frasch, E.F. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry 119, 115-119.
- Tsai, J.W., Hsu, S.T. & Chen, L.C. (1985). Bacteriocin-producing strains of P. solanacearum and their effect on development of bacterial wilt of tomato. Plant Protection Bulletin, Taiwan 27 267-278.
- Vaara, M., Vaara, T., Jensen, M., Helander., Nurminen, M., Rietschel, E.Th., & Makela, P.H. (1981). Characterization of the lipopolysaccharide from the polymyxin-resistant pmrA mutants of Salmonella typhimurium. FEBS Letters 129, 145-149.
- Van Alfen, N.K., McMillan, B.D., Turner, V. & Hess, W.M. (1983). Role of pit membranes in macromolecule induced wilt of plants. Plant physiology 73, 1020-1023.
- Van Alfen, N.K. (1989). Reassessment of plant wilt toxins. Annual Review of Phytopathology 27, 533-550.
- Van Alphen, L., Lugtenberg, B., Van Boxtel, R. & Verhoef, K. (1977) Architecture of the outer membrane of Escherichia coli K-12: action of phospholipase A₂ & C on wild-type strains and outer membrane mutants. Biochimica et Biophysica Acta 466, 257-268.
- Van der Ley, P., Bekkers, A., Van Meersbergen, J. & Tommassen, J. (1987). A comparative study of the phoE genes of three enterobacterial species. Implications for structure-function relationships in a pore-forming protein of the OM. European Journal of Biochemistry 164, 469-475.
- Van Regenmortel, M.H.V. (1982). Serology and Immunochemistry of plant viruses. Academic Press, New York. 302pp.
- Van Vuurde, J.W.L., (1987). New approach in detecting

- phytopathogenic bacteria by combined immunoisolation and immunoidentification assays. Bulletin OEPP/EPPO Bulletin 17, 139-148.
- Van Vuurde, J.W.L., van den Bovenkamp, G.W. & Birnbaum, Y. (1983). Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of Pseudomonas syringae pv. phaseolicola and Xanthomonas campestris pv. phaseoli in bean seed. Seed Science Technology 11, 547-559.
- Van Weemen, B.K. & Schuurs, A.H.W.M. (1971). Immunoassay using antigen-enzyme conjugates. FEBS Letters 15, 232-236.
- Vidaver, A.K. (1982) The plant pathogenic Corynebacteria. Annual Review of Microbiology 36, 495-517.
- Vidaver, A.K. (1983). Bacteriocins: The lure and the reality. Plant Disease 67 471-475.
- Vines, A.E. & Rees, N. (1972). Plant and Animal Biology Volume 1. London. Pitman.
- Vogt, R.F.Jr., Phillips, D.L., Henderson, L.O., Volpi, A., Lakeman, A.D., Pereira, L. & Stagno, S. (1983). Monoclonal antibodies for rapid diagnosis and typing of genital herpes infectious during pregnancy. American Journal of Obstetrics and Gynaecology 146, 813-815.
- Voogt, C.E., van der Stel, J.J. & Jacobs, J.J.J.A.A. (1980). On the mutagenic action of enzyme immunoassay substrates. Journal of Immunological Methods 36, 55-61.
- Vruggink, H. (1978). Enzyme-linked immunosorbent assay (ELISA) in the serodiagnosis of plant pathogenic bacteria. Proceedings of the 4th International Conference on Plant Pathogenic Bacteria, Angers 307-310.
- Vruggink, H. & De Boer, S.H. (1978). Detection of Erwinia carotovora var. atroseptica in potato tubers with immunofluorescence following induction of decay. Potato Research 21, 225-229.

- Vruggink, H. & Maas Geesteranus, H.P. (1975). Serological recognition of Erwinia carotovora var. atroseptica, the causal organism of potato blackleg. Potato Research 18, 546-555.
- Wall, R. & Kuehl, M. (1983). Biosynthesis and regulation of immunoglobulins. Annual Review of Immunology 1, 393-422.
- Waller J.M. & Sitepu, D. (1971). Sumatra Disease of cloves in Indonesia. PANS 21, 141-147.
- Wallis, F.M. & Truter, S. (1978). Histopathology of tomato plants infected with P. solanacearum with emphasis on ultrastructure. Physiological Plant Pathology 13 307-317.
- Walls, E.V., Lam, K. & Crawford, D.H. (1988). Production of human monoclonal antibodies using Epstein-Barr virus. In : Clinical applications of monoclonal antibodies. Hubbard, R. & Marks, V. (Eds.). New York. Plenum Press. 258pp.
- Ward, E.S., Gussow, D., Griffiths, A.D., Jones, P.T. & Winter, G. (1989). Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli. Nature 341, 544-546.
- Wells, J.M., Raju, B.C. & Nyland, G. (1983). Isolation, culture and pathogenicity of the bacterium causing phony disease of peach. Phytopathology 73 859-862.
- Wells, J.M., Raju, B.C., Hung, H., Weisburg, W.G., Mandelco-Paul, L. & Brenner, D.J. (1987). Xylella fastidiosa gen. nov., sp. nov: Gram-negative, xylem-limited, fastidious plant bacteria related to Xanthomonas spp.. International Journal of Systematic Bacteriology 37, 136-143.
- Whatley, M.H., Hunter, N., Cantrell, M.A., Hendrick, C., Keegstru, K. & Sequeira, L. (1980). LPS composition of the wilt pathogen P. solanacearum. Plant Physiology 65 557-559.
- Whitfield, W. & Spierto, F.W. (1987). Quantitative

- differences among various proteins as blocking agents for ELISA microtiter plates. Journal of Immunological Methods 101, 43-50.
- Williams, G. (1988). Novel antibody reagents: production and potential. Trends in Biotechnology 6, 36-42.
- Williams, P.H. (1980). Black Rot: A continuing threat to world crucifers. Plant Disease 64, 736-742.
- Wolf, R.T. & Nelson, P.E. (1969). An anatomical study of carnation stems with the carnation strain of E. chrysanthemi. Phytopathology 59 1802-1808.
- Wu, T.T. & Kabat, E.A. (1970). An analysis of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. Journal of Experimental Medicine. 132, 211-250.
- Xu, P., Iwata, M., Leong, S. & Sequeira, L. (1990). Highly virulent strains of P. solanacearum that are defective in extracellular polysaccharide production. Journal of Bacteriology 172, 3946-3951.
- Xu, P., Leong, S. & Sequeira, L. (1988). Molecular cloning of genes that specify virulence in P. solanacearum. Journal of Bacteriology 170, 617-622.
- Yelton, D.E., Diamond, B.A., Kwan, S.P. & Scharff, M.D. (1978). Fusion of mouse myeloma and spleen cells. Current Topics in Microbiology and Immunology 81 1-7.
- Yolken, R.H. (1982). Enzyme immunoassays for the detection of infectious agents in body fluids: current limitations and future prospects. Review of Infectious Diseases 4, 35-68.
- Yolken, R.H. & Stopa, P.J. (1980). Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. Journal of Clinical Microbiology 11, 546-551.

APPENDIX I - REAGENTS USED AND SUPPLIERS

MISCELLANEOUS CHEMICALS

Acetone AR	May and Baker Ltd
Citric Acid AR	F S A L a b o r a t o r y S u p p l i e s
di-Sodium hydrogen orthophosphate dihydrate AR ($\text{Na}_2 \text{HPO}_4 \cdot 2\text{H}_2\text{O}$)	BDH Chemicals Ltd
Ethanol (95% v/v)	F S A L a b o r a t o r y S u p p l i e s
Glycine AR	BDH Chemicals Ltd
Methanol	F S A L a b o r a t o r y S u p p l i e s
Potassium chloride AR	F S A L a b o r a t o r y S u p p l i e s
Potassium dihydrogen orthophosphate AR (KH_2PO_4)	F S A L a b o r a t o r y S u p p l i e s
Potassium Hydroxide AR	F S A L a b o r a t o r y S u p p l i e s
Sodium Acetate AR (anhydrous)	BDH Chemicals Ltd
Sodium Azide (NaN_3)	Sigma Chemical Co. Ltd
Sodium carbonate AR	F S A L a b o r a t o r y S u p p l i e s
Sodium Chloride AR	BDH Chemicals Ltd
Sodium hydrogen carbonate AR (NaHCO_3)	F S A L a b o r a t o r y S u p p l i e s
Sodium hydroxide pellets AR	May and Baker Ltd

CELL CULTURE REAGENTS

^{14}C -lysine	Amersham International
Dimethylsulphoxide	Sigma Chemical Co. Ltd
DMEM (without L-lysine)	Gibco Ltd
Dulbecco's Modification of Eagles Medium (1x) with 3.70 g/l sodium bicarbonate, without glutamine	Flow Laboratories
HAT Supplement (50x)	Gibco Ltd
HAza (50x)	Sigma Chemical Co. Ltd
HT (50x)	Gibco Ltd
Hypoxanthine (100x)	Gibco Ltd
L-Glutamine (200 mM)	Flow Laboratories
2-Mercaptoethanol (50 mM)	Gibco Ltd
MRC5 cell line	Flow Laboratories
Myoclon plus Foetal Calf Serum	Gibco Ltd
Penicillin/Streptomycin solution (10000 IU/ml : 10000 $\mu\text{g/ml}$)	Gibco Ltd
Polyethylene Glycol 1500 (50% w/v in 75 mM Hepes)	Boehringer Mannheim Ltd
X63/Ag8/653 cell line	Flow Laboratories

IMMUNOCHEMICALS AND IMMUNOASSAY REAGENTS

4-Chloro-1-Naphthol (30 mg tablets)	Sigma Chemical Co. Ltd
Freund's Complete Adjuvant (Bacto)	Difco Laboratories
Cyanogen Bromide Activated Sephadex 4B	Sigma Chemical Co. Ltd
Dried Milk Powder (Five Pints)	St. Ivel Ltd
Glutaraldehyde (25%, EM Grade)	Agar Scientific Ltd
Glycerol (AR)	Fisons Ltd
Goat anti-mouse IgG (H+L) -FITC conjugate	Sigma Chemical Co. Ltd
Goat anti-mouse IgG (H+L) -R-phycoerythrin conjugate	Sigma Chemical Co. Ltd
Goat anti-mouse IgG (H+L) -TRITC conjugate	Sigma Chemical Co. Ltd
6% v/v Hydrogen Peroxide solution	Sigma Chemical Co. Ltd
Incomplete Freund's Adjuvant (Bacto)	Difco Laboratories
Mouse Monoclonal Antibody isotyping kit	Bio Rad Laboratories
Nitrocellulose (0.2 µm pore size)	Schleicher and Schuell
Rat Monoclonal Antibody isotyping kit	Serotec Ltd
Sheep anti-mouse IgG (H+L) Horseradish Peroxidase conjugate	The Binding Site Ltd
Sheep anti-mouse IgG (H+L) TRITC conjugate	The Binding Site Ltd
Sheep anti-rabbit IgG (H+L) Horseradish Peroxidase conjugate	The Binding Site Ltd
Sheep anti-rat IgG (H+L) Horseradish Peroxidase conjugate	The Binding Site Ltd
Sulphuric Acid AR	FSA Laboratory Supplies
Teflon coated multiwell slides	Dynatech Laboratories
3,3',5,5'-Tetramethylbenzidine	Sigma Chemical Co. Ltd
Tween 20 (Polyoxyethylene- Sorbitan Monolaurate)	Sigma Chemical Co. Ltd

SDS-PAGE AND IMMUNOBLOTTING REAGENTS

Acrylamide/N-N'-Methylene-bis- acrylamide (37.5:1 (2.6%C) 30 g preweighed mixture)	Bio Rad Laboratories
Amido Black (Naphthol Blue Black)	Sigma Chemical Co. Ltd
Ammonium Persulphate (Electran)	BDH Chemicals Ltd
Bromophenol Blue	Sigma Chemical Co. Ltd
Coomassie Brilliant Blue R	Sigma Chemical Co. Ltd
Dithiothreitol	Sigma Chemical Co. Ltd
Formaldehyde (AR)	BDH Chemicals Ltd
Methyl Green	Sigma Chemical Co. Ltd
MOPS (3-[N-Morpholino] propane sulphonic acid) Free Acid	Sigma Chemical Co. Ltd
Protein Molecular Weight Standards	Sigma Chemical Co. Ltd

Proteinase K	
(from <u>Tritirachium album</u>) Type X1	Sigma Chemical Co. Ltd
Silver Nitrate (AR)	BDH Chemicals Ltd
Sodium Dodecyl Sulphate (SDS)	BDH Chemicals Ltd
Sucrose (AR)	F S A L a b o r a t o r y
Supplies	
N,N,N',N'-tetramethylethylenediamine (TEMED)	BDH Chemicals Ltd
Trizma Base (Tris [hydroxymethyl] aminomethane)	Sigma Chemical Co. Ltd

APPENDIX II - BUFFERS, MEDIA ETC.

All water used in the preparation of solutions was either deionized and distilled or double distilled.

CELL CULTURE MEDIA

Agar Medium for Soft Agar Cloning

0.5 g of agar (Difco Bacto) was dissolved in 20 ml dH_2O , sterilized by autoclaving (15 p.s.i. for 15 minutes), and allowed to cool to 45°C (in a water bath). The cooled agar was poured into 80 ml of culture medium (20 ml double strength DMEM + 10 ml FCS + 50 ml DMEM/20) at 45°C .

12 ml aliquots of the cloning agar mix were poured into petri-dishes and allow to cool.

1 ml aliquots of the cloning agar were added to the serially diluted cells.

Selective Media for Hybridomas

Selective media were made by the addition of the appropriate amount of concentrated supplement to give either a double strength solution (HAT and HAZa) or a single strength solution (HT and hypoxanthine) in DMEM/20.

Radioactive Incorporation Medium for Isotyping of Rat mAbs

DMEM (without L-lysine)	9 ml
Dialysed FCS*	0.5 ml
^{14}C -lysine ($50\mu\text{Ci/ml}$)	1 ml

* : FCS was dialysed against double distilled water. After dialysis, 1/9th volume of 10 times balanced saline solution was added.

MICROBIOLOGICAL MEDIA

All media were sterilized by autoclaving for 15 minutes at 15 p.s.i. (120°C).

Casamino Acids Medium (CA+)

Acid casein hydrolysate (Oxoid)	7.5 g
Sucrose	2 g
K ₂ HPO ₄	0.5 g
MgSO ₄ 7H ₂ O	0.25 g

made up to 1 litre with dH₂O.

After autoclaving add ferric ammonium citrate (2.5% w/v, filter sterilized) at 1 ml/100 ml.

For solid medium add 15 g/litre of Difco agar.

Sucrose Peptone Medium

Sucrose	20 g
Peptone (Difco)	5 g
K ₂ HPO ₄	0.5 g
MgSO ₄ 7H ₂ O	0.25 g

made up to 1 litre with dH₂O, and adjusted to pH7.2-7.4.

For solid medium add 15 g/litre of Difco agar.

Periwinkle Wilt Medium

Peptone (Difco)	4 g
Tryptone	1 g
K ₂ HPO ₄	1.2 g
KH ₂ PO ₄	1 g
MgSO ₄ 7H ₂ O	0.4 g
Phenol red	20 mg

made up to 1 litre with dH₂O.

After autoclaving, add ferric ammonium citrate (2.5% w/v) at 1 ml/100 ml, bovine serum albumin (0.6% w/v) and L-glutamine (0.4% w/v) (all filter sterilized).

For solid medium add 15 g/litre of Difco agar.

IMMUNOCHEMICAL AND IMMUNOASSAY BUFFERS

Physiological Saline Solution

Dissolve 0.85 g of sodium chloride in 100 ml of dH₂O.

Saturated Ammonium Sulphate Solution pH7.2

Dissolve 1000 g of ammonium sulphate in 1000 ml dH₂O at 50°C. Stand overnight at room temperature, and adjust the pH to 7.2 with dilute ammonia solution (use 1/100 diluted samples to test the pH).

0.05M Sodium Carbonate Buffer pH9.6

Sodium Carbonate (Na ₂ CO ₃)	1.59 g
Sodium Hydrogen Carbonate (NaHCO ₃)	2.93 g
made up to 1 litre with dH ₂ O.	

0.1M Citrate Buffer pH3 and pH5

Prepare 0.1M solutions of:

Citric acid (C ₆ H ₈ O ₇ 1H ₂ O)	21 g/l
and	
Di-sodium hydrogen orthophosphate	17.8 g/l

For pH5 mix equal volumes of the two solutions.
For pH3, titrate the phosphate solution with the citric acid solution.

1.5M (10x) Phosphate Buffered Saline pH7.4

Sodium Chloride (NaCl)	80 g
Potassium Dihydrogen Orthophosphate (KH ₂ PO ₄)	2 g
di-Sodium Hydrogen Orthophosphate dihydrate (Na ₂ HPO ₄ ·2H ₂ O)	29 g
Potassium chloride (KCl)	2 g
made up to 1 litre with dH ₂ O.	

PBS+ 0.05% Tween 20

PBS (10x)	100 ml
10% Tween 20 (in dH ₂ O)	5 ml
made up to 1 litre with dH ₂ O.	

Horseradish Peroxidase Soluble Substrate Solution for ELISA

Stock solutions:

- A. 1M sodium acetate adjusted to pH5.8 with citric acid;
- B. 10 mg of 3,3',5,5'-tetramethylbenzidine dissolved in dimethylsulphoxide (1 ml) (stored frozen at 4°C);
- C. 6% v/v hydrogen peroxide

Just before use mix:

dH ₂ O	9 ml
Solution A.	1 ml
Solution B.	100 µl
Solution C.	10 µl

5% Dried Milk Powder (DMP) Solution (in PBS or PBSt)

Emulsify 5 g of DMP in a small volume of diluent to form a creamy paste and then add diluent to a final volume of 100 ml.

Horseradish Peroxidase Insoluble Substrate Solution for DIA

Dissolve 1 tablet (30 mg) of 4-Chloro-1-Naphthol in 10 ml of methanol (at 4°C). Add to 50 ml of PBS (1x). Add 30 µl of 6% v/v hydrogen peroxide.

0.1M Phosphate Buffered Glycerol pH7.6

Na ₂ HPO ₄ ·2H ₂ O	1.57 g
NaH ₂ PO ₄	0.15 g
Glycerol	50 ml
dH ₂ O	100 ml

Store at 4°C or add sodium azide to a final concentration of 0.02% w/v.

SDS-PAGE AND ELECTROBLOTTING

Separating Gel Buffer (3M Tris-HCl;pH8.8)

Dissolve 181.5 g Trizma base in 300 ml dH₂O. Adjust pH to 8.8 with 5M HCl. Make up to 500 ml with dH₂O. Degas before use.

Stacking Gel Buffer (0.5M Tris-HCl;pH6.8)

Dissolve 30.3 g of Trizma base in 400 ml dH₂O. Adjust pH to 6.8 with 5M HCl. Make up to 500 ml with dH₂O. Degas before use.

1.5% w/v Ammonium Persulphate Solution

Dissolve 0.15 g ammonium persulphate (APS) in 10 ml of degassed dH₂O. Make up immediately prior to use.

10% w/v Sodium Dodecyl Sulphate (SDS)

Dissolve 10 g of SDS in dH₂O to 100 ml.

Electrode Buffer (25mM Tris-192mM Glycine, 0.1% SDS), pH8.3

Trizma base	3.03 g
Glycine	14.41 g
SDS	1 g

Make up to 1 litre in dH₂O.

SDS-PAGE Sample Buffer

dH ₂ O	4.4 ml
0.5M Tris-HCl; pH6.8	1 ml
Glycerol	0.8 ml
10% SDS	1.6 ml
Dithiothreitol	123 mg
0.05% Bromophenol blue	0.2 ml

0.1% w/v Coomassie Blue Stain

Dissolve 0.1 g Coomassie Brilliant Blue R in 100 ml of methanol:acetic acid:dH₂O (4:1:5).

Coomassie Blue Destain

Methanol:acetic acid:dH₂O (4:1:5).

0.5% w/v Amido Black Stain

Dissolve 0.5 g amido black in dH₂O:methanol:acetic acid (45:50:5).

Amido Black Destain

dH₂O:methanol:acetic acid (45:50:5).

0.25% w/v Methyl Green

dH ₂ O	9 ml
Glycerol	1 ml
Methyl Green	0.025 g

Electrotransfer buffer (2mM sodium acetate, 5mM morpholinopropanesulphonic acid (MOPS) pH7.5, in 20% v/v ethanol)

Sodium Acetate 0.82 g

MOPS 5.23 g

Dissolve in 4 litres of dH₂O). Adjust pH to 7.5 with 5M sodium hydroxide. Add 1 litre of 95% ethanol.

The Preparation of 5-16% SDS-PAGE Gradient Gels

Separating gel solutions:

	<u>Amount added (ml)</u>	
	<u>5%</u>	<u>16%</u>
dH ₂ O	10.8	3.6
3M Tris-HCl pH8.8	2	2
10%SDS	0.16	0.16
Acrylamide/bisacrylamide	2.66	8.52
APS	0.372	0.372
Sucrose	-	2.4 g
TEMED	5 µl	5 µl

Stacking gel solution:

dH ₂ O	11.3 ml
0.5M Tris pH6.8	5 ml
Acrylamide/bisacrylamide	2.5 ml
1.5%APS	1 ml
10%SDS	0.2 ml
TEMED	12 µl

- Add 73 ml of degassed dH₂O to 30 g of preweighed, Bio-Rad acrylamide/bisacrylamide mixture (37.5%), to give a final volume of 100 ml;
- Prepare 5% and 16% acrylamide solutions;
- Pour a 5-16% gradient gel using a gradient mixer;
- Overlay the gel with 0.5M Tris HCl; pH8.8 and leave overnight;
- Remove overlay and add stacking gel solution (approximately 7.5 ml needed for Protean II gels);
- Insert well forming comb, allow gel to set, and use immediately.

Silver Staining of PAGE Gels

Prepare the following solutions in dH₂O:

- A. Ethanol:acetic acid;dH₂O (25:10:65);
- B. Dithiothreitol, 5 µl/ml
- C. 0.1% w/v silver nitrate;
- D. 0.02% formaldehyde (0.1 ml of 40% formaldehyde in 200 ml);
- E. 3% w/v sodium carbonate;
- F. 2.3M Citric acid.

Staining Procedure

- a). Fix the gel in solution A for 2 hours
- b). Wash gel in dH₂O for 1 hour
- c). Soak gel in solution B for 2 hours
- d). Remove solution B and add solution C. Soak for 2 hours
- e). Rinse gel quickly with two changes of dH₂O
- f). Develop in a mixture of D (0.1 ml) and E (200 ml) until stained bands are observed
- g). Stop the reaction by the addition of 10 ml of E, and soak for a further 30 minutes
- h). Store gel in dH₂O.